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HANDBOOK FOR EVALUATING WATER BACTERIOLOGICAL LABORATORIES

Municipal Environmental Research Laboratory

August 1975

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HANDBOOK FOR EVALUATING WATER BACTERIOLOGICAL LABORATORIES

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U.S. ENVIRONMENTAL PROTECTION AGENCY

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The material included in this Handbook is designed and intended to provide a comprehensive source of information and reference for the evaluation of laboratories involved in bacteriological testing of potable water supplies and their sources. All aspects of the laboratory operation are considered--material and media preparation, equipment needs and specifications, sample collection and handling, bacteriological methodology, quality control considerations, laboratory management, and the qualifications and responsibilities of the survey officer. The purpose of the Handbook is to assist the laboratory survey officer, laboratory director, and senior bacteriologist in charge of the water program to evaluate the many aspects of the laboratory that are involved in attaining reliable data.

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EVALUATING WATER BACTERIOLOGICAL LABORATORIES
Second Edition

Edwin E. Geldreich
Microbiological Quality Control
Water Supply Research Division
Municipal Environmental Research Laboratory
Cincinnati, Ohio 45268

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OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
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The Municipal Environmental Research Laboratory, U.S. Environmental Protection Agency, has reviewed the report and approved its publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

The Safe Drinking Water Act (Title XIX of the Public Health Act), enacted into law on December 16, 1974, requires the Administrator of the U.S. Environmental Protection Agency to promulgate a set of primary drinking water regulations. These regulations, which will apply to all public drinking water systems as defined in "The Act" shall

- specify contaminants that may have any adverse effect on the health of persons;
- specify a maximum contaminant level or a treatment technique;
- contain criteria and procedures to ensure a safe drinking water supply.

The various states will be responsible for ensuring that the local water supply utilities meet the primary drinking water regulations. Therefore, the U.S. Environmental Protection Agency's State Laboratory Certification Program has become an integral part of carrying out the provisions of the Safe Drinking Water Act.

This report, developed by the Water Supply Research Division, Municipal Environmental Research Laboratory, as part of our continuing responsibilities for the certification of state water supply laboratories, is an update and expansion of a similar document published by the Public Health Service in 1966. The effort contained in this supportive document represents part of the U.S. Environmental Protection Agency's total quality assurance and laboratory certification program in the areas of water pollution abatement and water supply protection. The Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, is responsible for developing certification criteria and methods manuals for both water and wastewater laboratories and is responsible for coordinating all Federal involvements in the total water quality assurance and laboratory certification programs.

A. W. Breidenbach, Ph.D.
Director
Municipal Environmental
Research Laboratory

ABSTRACT

The material included in this Handbook is designed and intended to provide a comprehensive source of information and reference for the evaluation of laboratories involved in bacteriological testing of potable water supplies and their sources. The information is based upon more than 15 years experience by the author in bacteriological laboratory surveys and observations of laboratory practices in water examination throughout this Nation.

The Handbook covers all aspects of the laboratory operation including material and media preparation, equipment needs and specifications, sample collection and handling, bacteriological methodology, quality control considerations, laboratory management, and the survey officer's qualifications and responsibilities.

The purpose of this Handbook is to assist the laboratory survey officer, laboratory director, and senior bacteriologist in charge of the water program to evaluate the many aspects of the laboratory that are involved in attaining reliable data.

PREFACE

The intensified concern with potable water quality and the development of criteria and standards for various classes of natural water are reflected in increased requests for more laboratory analyses. These requests now include not only the traditional total coliform procedure used to monitor contamination breakthroughs into finished waters, but also standard plate counts to detect water quality deterioration in distribution networks. Recreational water quality criteria include fecal coliform limits, and epidemiological investigations may require examinations for some specific waterborne pathogens. Thus, the bacteriological laboratory today must have capabilities for expanded examinations.

In 1943, L. A. Black of the U.S. Public Health Service, developed a survey form for water bacteriology laboratories, which was utilized by the Public Health Service personnel during periodic evaluations of state laboratories. Additionally the form was used by various state survey officers in the evaluation of those laboratories within their respective states that were involved in the examination of water. A similar check of state water chemistry laboratories was not made, however, since only a few states performed routine chemical analyses. In fact, even today, some states do few or no routine water chemical determinations and the remainder do less than an adequate job of surveillance. In an effort to improve this situation, the development of a water chemistry survey form was initiated in July 1969 and is now being used to evaluate state and Federal water chemistry laboratories by specialists in chemistry.

The demand for expanded laboratory involvement by various environmental agencies has created a need for this second edition of the manual *Evaluation of Water Laboratories* first published by the Public Health Service in 1966. This document was the product of prepared notes and ideas developed by both Harold F. Clark and Edwin E. Geldreich in their assignments to evaluate bacteriological laboratories responsible for the examination of water supplies. Many of their laboratory research developments in methodology have since been adopted by *Standard Methods for the Examination of Water and Wastewater*.

Over 5,000 copies of the first edition were circulated to bacteriologists, chemists, sanitary engineers, water plant management personnel, university professors, college students, and numerous foreign scientific centers concerned with laboratory quality control in their countries. As a result of the unforeseen demand for a modest attempt to supply guideline assistance to those persons involved in laboratory evaluations, the supply of the first edition is now depleted.

While preparing the second edition, a more general coverage of laboratory practice beyond the scope or intent of *Standard Methods for Examination of Water and Wastewater* was sought. This new approach was also used in revising the bacteriological survey form (EPA-103) to increase its flexibility and make it more useful in evaluating laboratories that examine stream and/or marine pollution samples in addition to potable waters. In developing both the survey form and the handbook, the intent was to present guidelines for conformity with *Standard Methods for the Examination of Water and Wastewater*, U.S. Environmental Protection Agency methods manuals, and other generally accepted laboratory practices. The underlying goal is to facilitate the collection of data having the greatest sensitivity, reliability and precision whether for monitoring potable and recreational water quality or for enforcement actions concerned with water quality degradation.

Edwin E. Geldreich
June 1975

ACKNOWLEDGMENTS

No study of this scope could be possible without the assistance of many state sanitary engineers, water-plant managers, laboratory directors, state survey officers, bacteriologists, sanitarians, and technicians who share a sincere interest and concern about potable water supplies and the meaningful measurement of bacteriological quality. The willingness of this multidiscipline group to discuss openly their problems, make records available for analysis, and correct recognized deviations in procedures conscientiously is greatly appreciated.

Special acknowledgements must also be given to Dr. Harry D. Nash, Dr. Donald J. Reasoner, and Mr. Raymond H. Taylor for their critical review of technical material and suggestions on narrative structure; to Mrs. Virginia Maphet for the difficult and precise task of manuscript preparation; to my wife, Detta, for proofreading copy during various stages of manuscript development; and to Mrs. Marion Curry for much appreciated editorial assistance in the evolution of the Handbook.

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CHAPTER I

INTRODUCTION TO LABORATORY EVALUATION

It is essential that laboratory methods be adopted that are reliable and produce uniform results in all laboratories involved in monitoring this Nation's 40,000 public water systems and the 10 million individual water supplies and the 200,000 water supplies serving the traveling public.

Analysis of data available to the U.S. Environmental Protection Agency (EPA) laboratory evaluation program indicates that state health, state environmental, city-county health, municipal water treatment, and private laboratories are examining approximately 3.5 million samples annually from this Nation's public and private water supplies and are gathering monitoring data on natural waters relative to state and Federal standards for a variety of water quality uses. An estimated million additional samples are analyzed by local laboratories in quality control monitoring of industrial and municipal waste discharges as required in the National Pollution Discharge Elimination System.

Data developed from these examinations must be reliable and beyond reproach when used in judgment of technical operations in water treatment or in legal action involving public health hazards. For these reasons, it is desirable to use a generally accepted set of standard test methods that are acknowledged by the scientific community as representing the best available procedures. The need to develop a unified approach to the examination of water quality was recognized in 1905 with the publication of the first edition of *Standard Methods of Water Analysis*. New editions of this reference appearing through the intervening years recognize a continuing need to reevaluate recommended procedures in response to new research developments.

Current editions of *Standard Methods for the Examination of Water and Wastewater* (1) (*Standard Methods*) receive legal acceptance at all levels of state and Federal court systems. By government regulation (2), all analyses of drinking water and water supply systems used by carriers and others subject to Federal quarantine regulations must conform with provisions of the current edition of the *Standard Methods* reference. One of the mission responsibilities of EPA's Water Supply Research Laboratory is to ensure that all laboratories follow proper application of *Standard Methods* in the examination of potable waters. Since this program includes not only state health laboratories, but also county and city health laboratories, municipal water plant laboratories, hospital, and university and private laboratories, there is a need for assistance at the state level in maintaining the extensive coverage of all laboratories involved.

Traditionally, the Federal water supply program has approved the state laboratories, which in turn, through qualified state laboratory survey officers, certify the local laboratories within each state. On occasion, the

Federal water program performs cross-section studies of the laboratory service within a given state to ascertain the quality of work being performed not only by the large state laboratory system but also by the small water plant laboratories that are checking finished water and quality throughout the distribution system. The ultimate goal is to upgrade the quality of data in all water plant laboratories so that it is acceptable as part of the official monitoring of public water supplies. At present, the data obtained by many small water plant laboratories cannot be used as official data because of questionable application of recommended procedures. Thus the state laboratory service is burdened with the complete monitoring requirements for all official samples examined monthly from each public water supply.

THE APPROACH TO LABORATORY EVALUATION

The laboratory survey officer should view the evaluation as a conference relating to methods and procedures recommended in *Standard Methods* and appropriate EPA Methods Manuals (3-5), emphasizing the need and importance for standard procedures that will produce reliable data, comparable to similar data from other laboratories. Certainly, endorsement of the laboratory as being approved by the state or Federal government does bring significant prestige, and discussion with recognized experts in water analyses affords the opportunity for increased technician knowledge. This attitude yields much better results with the majority of the laboratories than does an attitude that emphasizes the regulatory activities of the visit.

PROGRAM OBJECTIVES

The objectives of a laboratory evaluation are to improve the quality of technical procedures so that the data compiled are reliable and to ensure that the water consumer and recreational water user are provided the greatest possible health protection. Techniques must always remain as sensitive as the state-of-the-art permits. This is of particular importance in the continued monitoring for the low levels of coliform bacteria that could signal the occurrence of possible contamination by pathogenic microorganisms. Technicians must always attach equal importance to every potable water examination, regardless of the source or the frequency of negative results. Monotony of negative results tends to breed technical carelessness that can quickly lead to bad habits and deviations from standard procedures. Although occasional deviations in technique may in themselves be insignificant, the cumulative effect of several deviations decreases test sensitivity and adversely reflects on data reliability. Failure to detect low levels of coliform organisms obviously poses a potential health hazard to consumers of such water.

Deviations in laboratory procedures will continue as a result of such factors as attempted shortcuts, ignorance of technical procedures, inexperience in new methods, equipment failures, inadequate facilities, technical carelessness, shifts of competent personnel to other laboratory assignments, and lack of interest in this phase of public health bacteriology. Thus, there exists a continuing need for laboratory evaluation services, both at the state and the municipal levels, to hold number of deviations to an absolute minimum (6, 7). For the purposes of estimating cost for this activity, 5 man-days are required per state or regional laboratory as contrasted with 3 man-days required for this same service at small local laboratories where testing capabilities are more limited. These time/cost studies should include survey preparation, travel time, per diem, site visits, and report preparation (8).

The optimum frequency of laboratory evaluations at the state level appears to be every 3 years. Visits at more frequent intervals are of little value to either the staff or the program; whereas, the longer the interval, the more deviations observed. However, where there are major difficulties or where there is a large turnover of laboratory personnel, evaluations must be performed at more frequent intervals, depending on the individual situation. For these reasons, EPA's Water Supply Division recommends that these laboratory evaluations should be accomplished at least every 3 years.

STATE WATER LABORATORY EVALUATION PROGRAM

The key to expanding the network of qualified laboratories monitoring public water supply quality throughout the Nation is in an effective and vigorously pursued certification program at both the Federal and state levels. The Federal program pioneered the development of the in-depth laboratory survey approach over 40 years ago and, through the years, has encouraged all states to formulate or expand their own certification programs (9).

As a result of these experiences, a protocol has emerged that is being used with some variations by many state laboratory evaluation groups. The first step is to establish an inventory of all laboratories known to be examining water. This exploratory list should include laboratories in the state; county, city health, and environmental protection departments; universities; and water and sewage plants, plus those commercial laboratories that advertise such services. Inquiry by letter or telephone is then made to determine the extent of the services available. The initial contact must establish what microbiological testing is being performed, type of waters examined, use of most probable number (MPN) or membrane filter (MF) procedure, and the availability of essential equipment items, including a copy of the current edition of *Standard Methods*. If it is established that the laboratory has all of the essential equipment and is using the recommended procedures, then the state survey officer should schedule the first on-site evaluation within 3 months of the initial contact. During this interval, a copy of the U.S. Environmental Protection Agency Bacteriological Survey Form is then sent to the laboratory personnel for a self-appraisal of their water program. Where it becomes evident that the laboratory does not have the required expertise, their designated personnel are generally invited to visit the state laboratory for several days to receive necessary bench training. These persons should also be encouraged to participate in a regional EPA laboratory training course or possibly receive individual training on specific techniques in water bacteriology.

Following the on-site survey, a copy of the evaluation report must be sent to the participating laboratory. If the laboratory is approved, a certificate with expiration date and a registration number should be issued. The certificate should state that the named laboratory has met the requirements and recommendations of the state agency and the EPA and is, therefore, authorized to perform specified bacteriological examinations of water. Certificates should then be reissued every 2 or 3 years following a satisfactory on-site laboratory evaluation.

There may be occasions when private laboratories in adjacent states may request certification for purposes of testing waters within the state. Reciprocal certification is feasible if the adjacent state survey officer can provide a copy of his evaluation report including a statement of satisfactory laboratory certification status. To this reciprocal agreement must be added a statement that laboratory data developed by the out-of-state laboratory are acceptable as official data only where sample transit times for potable waters do not exceed 30 hours; or where maximum transit times for stream and effluent water quality measurements do not exceed 6 hours.

During the interval between recertification, the state evaluation program should develop a bacteriological split-sampling protocol to test laboratory proficiency and to reaffirm the continuing production of reliable data (10-13). Pure cultures might afford some measure of testing laboratory proficiency, but they will not be representative of the interplay of mixed microbial flora common to a natural water sample. Possibly a mixed microbial flora could be created in an artificial test sample that would be representative of microbial interferences that produce some of the characteristic interferences inherent to both the multiple tube and MF procedures. Standard plate count agars should be tested for optimum recovery and, by use of organisms that produce only small colonies, a test of technician counting proficiency could be made.

A complete study of a laboratory including evaluation of procedures, equipment, and research; consultations with the laboratory personnel; and a review of findings can rarely be done in less than 4 hours. In evaluating a new laboratory for the first time, extra time should be allotted to orient the staff and management to the benefits of the evaluation and the desired program objectives. Each state evaluation program should maintain a current list of the laboratories having the capability for bacteriological examination of water and include those approved or certified and any laboratories provisionally approved or in noncompliance. Survey frequency should be on a 2-year basis when the laboratory procedures are acceptable but on a 6-month to 1-year basis for those laboratories receiving a provisional approval status. Immediate reevaluation becomes mandatory in the small local, private, or commercial laboratory upon a change in the laboratory director's or chief laboratory technician's position.

The designated state laboratory survey officer must be certified by a member of the Federal laboratory evaluation service. Certification is based upon knowledge of coliform detection methods, required laboratory apparatus, media requirements, and analysis of laboratory records during a joint visit of the designated state survey officer and the Federal counterpart. The state designate should be observed to have those qualities of temperament conducive to establishing a cooperative attitude among the laboratory personnel being reviewed without incurring resentment.

GENERAL STATUS OF LABORATORIES

In general, more current laboratory procedures, newer equipment and laboratory facilities, and more experienced personnel are found in state laboratories than are found in many municipal laboratories. The major obstacles are related directly to limited financial budgets that prevent purchasing necessary replacement equipment and low salary levels that don't attract technicians with the desired academic background. These two difficulties can contribute to poor quality laboratory service. A 1971 analysis of laboratory evaluation reports on 69 state, regional, local health laboratories and 93 municipal laboratories indicated an average of four deviations per state laboratory as contrasted to an average of six deviations per municipal laboratory. Many of the deviations observed in municipal laboratories reflected the need for equipment replacement (autoclaves, incubators, pH meters, analytical balances, water stills) and the lack of attention given to procedural details used in the examination of potable water. It was particularly disturbing to note that 12 municipal laboratories did not have the current edition of *Standard Methods* available for reference to the acceptable techniques.

INITIATING A REQUEST FOR EVALUATION

Most requests for a laboratory evaluation originate from laboratories that previously benefited from this service and have taken pride in receiving certification. In other instances, interest in a program review originates from laboratory personnel seeking advice on a major changeover in choice of *Standard Methods'* tests or because of changes in laboratory personnel. Infrequently, requests for a review of laboratory procedure originate because of discrepancies in data obtained from different laboratories involved in some overlap monitoring of municipal supplies or surveillance of bathing water quality.

An upsurge in laboratory evaluation requests to the Federal water supply program relate to a growing number of state water supply divisions interested in obtaining in-depth studies of all elements of their program activities. These special analyses of laboratory service include large and small water plant laboratories that may or may not have been evaluated by the state, plus study of the state branch laboratory system and water supply surveillance program. The net result is the necessity to evaluate more than just the central or state laboratory.

In initiating a request, local laboratories should transmit a written request through supervisory channels to the director of state laboratories, attention of the water laboratory survey officer. State health laboratories requesting a similar review of their water laboratory section should address their requests to the Regional Administrator, U.S. Environmental Protection Agency. Dates for evaluation visits at both the state and national level are usually grouped by geographical areas to conserve both staff time and travel money. With an emergency request, however, every effort will be made by the survey officer to respond as promptly as possible.

CONDUCTING THE EVALUATION

The laboratory survey should be scheduled in advance and at a time agreeable with both the consultant and laboratory personnel. Unscheduled surveys may be necessary under certain circumstances. For example, if a laboratory is placed on provisional or prohibited status, the subsequent survey should be scheduled. If, however, it is necessary to retain the laboratory on provisional or prohibited status, the survey officer could then exercise the prerogative of making an unannounced visit for a progress report or for formulating a final decision on prohibiting any further official water examination in that facility.

Both the survey officer and laboratory personnel must cooperate by assuming certain responsibilities in order to gain maximum benefit from the survey. The laboratory should schedule sufficient water examinations so that all routine bacteriological procedures can be evaluated from the initial processing steps to the concluding phase of reading test results and recording data. All laboratory personnel involved in conducting any or all of the analyses should be present during the survey and be prepared to discuss all aspects of their operations. Records and bench sheets should be available for inspection and a statistical summary prepared to show the number of tests performed, types of procedures used, and types of water samples examined each month.

The survey officer is responsible for examining procedures and equipment in detail to determine their compliance with *Standard Methods* or other acceptable laboratory practices. The survey officer is expected to explain any deficiencies observed in the records, such as insufficient samples per month, inadequate sampling of the distribution network, sample transit time, and response to unsatisfactory samples. When technical procedures are questionable, the consultant should explain the deviation and demonstrate the proper procedure—and should also be prepared to offer assistance concerning economics relating to testing time, available bench space, utilities, commercial media, presterilized and disposable items, and instrumentation aids.

It is hoped that the execution of these responsibilities will result in a rapport between the laboratory staff and consultant that will motivate an open discussion beneficial to everyone.

USING THE SURVEY FORM

Systematic coverage of the many technical procedures, equipment items, chemical reagents, media requirements, and allied activities that are essential elements of the water laboratory can best be reviewed through the use of a survey form. Rather than considering the survey form as a check list of laboratory activities, it should serve as a guideline to the creation of a specific description of the laboratory and its functions, work load, and deficiencies.

The bacteriological survey form should be filled out during the laboratory program review. Each item should be investigated as to its application, be it obvious or not. The marking code consists of an "X" for deviation, an "O" for an item that does not apply to the laboratory being reviewed, and a "U" for items not determined. These marks should be placed in the space provided adjacent to the appropriate item. During the program review, the survey officer will find it desirable to check-off the items as they are observed since it is often more convenient to follow the daily laboratory routine rather than to follow the order given on the survey form. All information requested, such as number of tests per year for each procedure, media lot numbers, and brand and model of equipment should be entered in the appropriate space on the form.

All the information recorded on this survey form should be used to formulate an oral report by the survey officer in a "wrap-up" conference held at the conclusion of the visit and to prepare a narrative report with specific comments and recommendations. Remember, the intent of the survey form is to serve as a guideline for complete coverage of the laboratory activities and not as a grading sheet for answers supplied by the laboratory staff.

REVIEW CONFERENCE

Each deviation observed during the laboratory evaluation should be discussed at the time it is observed. The discussion should include the deviation, its effect on the validity of results, remedial action, and reasons justifying the change in procedures. The final portion of each laboratory evaluation visit is devoted to an informal presentation of material to be covered in the narrative report. Generally, these program reviews are made to the laboratory director, chief bacteriologist in charge of the water program, and a representative of the water supply engineering staff. The presence of regional engineering staff members from the Federal water programs should be encouraged whenever the evaluation involves public water supply monitoring or water quality standards on interstate waterways.

Effective use of the time devoted to a review conference with the laboratory director requires that the laboratory survey officer prepare notes in a logical order for presentation. One suggested approach would be to discuss related items in a systematic order, such as:

1. Sampling and monitoring response
2. Laboratory equipment and instrumentation
3. Laboratory materials preparation and sterilization
4. Media
5. Multiple tube procedures
6. Membrane filter procedures
7. Supplementary bacteriological methods
8. Quality control program
9. Data processing and records
10. Laboratory safety
11. Laboratory facilities and staff
12. Summary comments and recommendations

These comments should not only be presented in a clear, orderly fashion but also be documented with illustrations from the records that underscore specific deviations from acceptable practice.

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GUIDELINES ON EVALUATING LABORATORIES

Program Objectives

Improve quality of laboratory data .... ________

Approve techniques based on current edition of *Standard Methods* or generally acknowledged good laboratory practice .... ________

Upgrade laboratory procedures so that data obtained in all laboratories can become part of official record .... ________

Minimize the number of deviations .... ________

Laboratory Evaluation Service

Federal program evaluates Federal, state, and selected local laboratories on a 3-year basis .... ________

State program evaluates all intra-state laboratories .... ________

State conducts survey on a ______________ year basis .... ________

State survey officer (Name) ____

Status of State laboratory evaluation service .... ________

Total:

______________ labs known to examine water

______________ approved laboratories

______________ provisionally approved laboratories

______________ nonapproved laboratories

Split sampling program supplements on-site survey .... ________

Conducting the Evaluation

Visit at mutually agreeable time unless laboratory is on a provisional or prohibited status .... ________

Variety of water examinations scheduled during the survey .... ________

Water program staff available during the survey for discussion of procedures ________

Records, laboratory work sheets, and year summary of tests performed available for inspection .... ________

Survey Officer's Responsibilities

Procedures and equipment used in the bacteriological examination of water examined .... ________

Records for sampling frequency, sampling program, sample transit time, and repeat sampling response inspected .... ________

Deviations in observed procedures discussed .... ________

Procedural changes, equipment and material needs, staffing requirements, and facility improvements recommended, as necessary .... ________

Survey form filled out during the visit .... ________

Results of the laboratory evaluation reviewed in conference with the Laboratory Director before concluding the visit .... ________

CHAPTER II

SAMPLING AND MONITORING RESPONSE

Essential links to meaningful laboratory data are the proper choice of the sampling location, strict adherence to proper sampling procedures, complete identification of the sample, and prompt transport of the sample to the laboratory. Coupled with these restrictions is the problem of getting a representative sample, be it potable water at various parts of the distribution system, or representative samples of the typical effluent quality of sewage or some industrial effluent.

The greatest obstacle to collection of a representative sample is in the possible lack of homogenity. This problem is most pronounced in sampling natural waters subjected to unpredictable inputs of storm water runoff and to industrial effluents whose quality may fluctuate severely because of varying industrial outputs or poorly managed treatment practices.

Even under the most favorable conditions, errors that relate to sampling are usually much greater than those in laboratory analyses. Unless samples are carefully selected and handled with care, taken at proper locations, promptly transported and laboratory processed, the results of these tests will be confusing, misleading, and detrimental to any monitoring program.

POTABLE WATERS

Compliance with the bacteriological requirements as prescribed in the Federal Drinking Water Standards must be based on a sampling program that includes examination of the finished water and a selection of distribution samples so that a systematic coverage of the distribution network is accomplished during each month. The essential consideration is the careful choice of distribution sample locations including dead-end sections to demonstrate that bacteriological quality is uniformly satisfactory throughout the network and to ensure that localized contamination does not occur through cross-connections, breaks in the distribution lines, or reduction in positive pressure. Sample locations may be public sites (police and fire stations, government office buildings, schools, bus and train stations, airports, community parks), commercial establishments (restaurants, gas stations, office buildings, industrial plants), private residences (single residences, apartment buildings, and townhouse complexes) and special sampling stations built into the distribution network. The establishment of an effective sampling program should be the joint responsibility of a local administrator (the water plant operator, health officer, or municipal engineer), the appropriate state engineering program, and the regional water supply representative of the EPA.

Sampling frequency, established by the Federal Drinking Water Standards, is based on a minimum monthly number requirement that is related

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to the population served by a given water supply. Thus, fewer bacteriological samples are required from smaller supplies. Ironically, the water systems serving populations of less than 50,000 people are more prone to show unsatisfactory bacteriological results (1). Data collected in 1969 from the Community Water Supply Study (2) of 969 public water supplies illustrate particular concern with bacteriological quality of potable supplies serving populations of 10,000 or less. Fifty percent of these smaller supplies had a history of unsatisfactory bacteriological results. The same study revealed that surveillance of 69 percent of the 969 water supplies was limited to only half the minimum number of monthly samples recommended by the Federal Drinking Water Standards; this reduced surveillance resulted, in part, from insufficient personnel and program funds.

Even though a sufficient number of monthly samples may be collected from small distribution systems, studies of sample records indicate that more than 75 percent of these required samples are taken from the same locations: the municipal building, the laboratory tap, the residence of some city official, and a favorite restaurant or tavern. Only occasional attempts may be made to obtain other samples that would more meaningfully measure water quality throughout the entire distribution system (1). It may be necessary to increase the number and location of monthly samples where supplies serve populations under 25,000 so that the entire network will be adequately monitored. Factors that must be considered in any modification of the sampling requirements include: frequency of unsatisfactory samples from supplies serving various population levels, repeat sampling and the time interval for repeat sampling, impact of peak water usage as related to seasonal shifts in populations, adequacy of treatment plant capacity, proper sampling of the distribution system, sample transit time to the laboratory, chlorine dosage, and raw water quality (some raw-water sources consistently contain more than 10,000 total coliforms per 100 ml).

NATURAL RECREATIONAL WATERS

Sampling locations for recreational areas should reflect the water quality within the entire recreational zone. Selected sites should include upstream peripheral areas and locations adjacent to drains or natural contours that would discharge stormwater collections or possible septic wastes from public restrooms, recreational buildings, and boat marinas. Sample collections taken in the swimming area should be obtained from a uniform depth of approximately 3 feet. Analysis of data taken from a series of small recreational lakes indicated that sampling depths of 3 and 6 feet did not produce any significant difference in bacteriological quality.

Base-line data on estuarine bathing water quality must include sampling at low tide, high tide and ebbtide. This initially intense sampling program will determine if any cyclic water quality deterioration occurs that must be controlled during the recreational season.

Sampling frequency should relate directly to the peak bathing period, which generally occurs in the afternoon. Preferably, daily samples should be collected during the recognized bathing season; minimum sampling should include Friday, Saturday, Sunday, and holidays—the periods of greatest recreational use. When limiting sampling to days of peak recreational use, a morning and afternoon sampling is desirable, particularly if the closing of bathing beaches is to be enforced on the basis of bacteriological quality of the water.

STREAM POLLUTION

Stream studies may be short-term high-intensity efforts involving the collection of substantial amounts of data on the variety of water quality criteria needed for some enforcement action. Choice of bacteriological sampling locations should include a base-line location upstream from the study area, industrial and municipal waste outfalls into the main stream study area, tributaries with a flow greater than 10 percent of the main stream, intake points for municipal water treatment plants and industrial needs, in addition to stream flow-time intervals and downstream recreational areas. Dispersion of effluents into the receiving stream may necessitate preliminary cross section studies to determine the completeness of mixing before final selection of sample stations (3). Where a tributary stream is involved, the sampling point should be near the confluence with the main stream; care must be taken not to sample backflow from the main stream. Sample collections are generally made from a boat or from bridges near critical study points. Locating sampling stations at the water treatment plant for collection of raw intake water may be useful but could yield lower bacterial densities than at the in-stream intake point when a raw water holding basin supply is, in fact, being measured. Frequency of sampling during a special field investigation of stream pollution should reflect conditions during normal industrial plant operation as well as during nonoperating hours, if possible. This can be accomplished by sampling every 4 to 6 hours and timing the same series to monitor slugs of pollutional discharges at each downstream location. Sampling intervals should be advanced 1 hour each day and be continued over a 7- to 10-day period.

Monitoring stream and lake water quality involves the establishment of sampling locations at critical sites that have been shown to reflect overall water quality. These sampling stations should be chosen with care since the resultant data may be used as base-line information on existing water qualities and as an early alert to the need for special field investigations involving specific pollutors or waste treatment deficiencies. Sampling frequency for monitoring stations may be seasonal for recreational waters, daily for water supply intake to the treatment plant, hourly where waste treatment control is erratic and effluents are discharged into shellfish harvesting areas, and continuous, if in the future, reuse water is used for potable water.

SEDIMENTS AND SLUDGES

An important aspect of long-term water quality conditions occurring in water supply reservoirs, in lakes, rivers and coastal waters for recreational purposes, and in shellfish growing waters may be found in the bacteriology of bottom sediments. These sediment deposits may provide a stable index of the general quality of the overlying water, particularly where there is great variability in the bacterial quality of the water (4).

Sampling frequency in reservoirs and lakes may be found to be related more to seasonal changes in water temperatures that cause overturn of stratified layers of differing water qualities. Bottom sediment changes in river and estuarine waters may be more erratic, being influenced by stormwater runoff, increased flow velocities, and sudden changes in the quality of effluent discharges.

Bacteriological examination of sludges from either water or sewage treatment processes are desirable to determine the impact of their disposal into receiving waters, ocean dumping, or burial in land-fill operations. Monitoring the bacteriological quality of sludge may also indicate the effectiveness of sewage treatment processes. Since the quality of sludges is subject to variations reflected in changes occurring in sewage composition and treatment responses, sampling frequency for this material may possibly better correlate with substantial changes in the biochemical oxygen demand in the effluent.

REPRESENTATIVE SAMPLES

Care must be exercised to collect samples representative of the water to be tested and to ensure that the sample does not become contaminated at the time of collection or before examination. Sterile sample bottles for bacteriological analysis must remain closed until the moment the sample is taken. At this time, ground glass stopper or screw cap and protective cover are then carefully removed. During the collecting procedure, care must be taken to avoid contact with the inner part of the closure or accidentally placing the closure on some dirty surface. The bottle is grasped at the base and filled nearly full without rinsing; ample air space is left for sample mixing. The closure should be replaced immediately and the protective cover, if employed, resecured around the bottle neck for additional protection. At this point, the sample must be properly identified or labeled, then placed in the appropriate container for delivery to the laboratory.

Ample Air Space

Adsorption of bacteria to particulate matter or to the inner surface of the sample bottle can occur between collection and examination of sample. Therefore, an ample air space must be left in the sample bottle at time of collection to permit adequate mixing for a resuspension of the bacterial population. Under no circumstance should the bacteriologist decant part of the sample in a full bottle to facilitate better mixing. This undesirable practice changes the bacterial density per unit volume and contributes to inaccurate bacteriological measurements. Samples without sufficient air space should be rejected, and a request should be made for a repeat sampling from that location. If this is not possible, carefully pour the entire sample into a larger sterile bottle and vigorously shake for complete mixing.

Minimum Sample Size

The minimum official sample volume cited in earlier editions of the Federal Drinking Water Standards and *Standard Methods* was either stated or implied to be 50 ml. This volume is necessary to inoculate each of a series of five lactose broth fermentation tubes with 10-ml portions of a potable water sample. Few laboratories routinely inoculate 100-ml portions in the multiple tube procedure because of the problems of preparing, handling, and incubating bottles large enough to culture 100-ml sample portions. The minimum sample volume collected for analysis from all classes of water, ranging from potable supplies, to stream, estuarine, and coastal waters, should be 100 ml, irrespective of the small volumes of sample actually utilized. Where special studies involve analyses for several bacterial indicators and a search for pathogens, the total sample volumes may involve 500 ml or more in a single sampling. Sample concentration techniques may be necessary where attempts are made to detect low levels of pathogen occurrences in water. Mack *et al.* (5) reported isolating poliovirus type II from a restaurant well-water supply only after 2.5-gallon samples were flocculated prior to centrifuging to concentrate the low density virus particles. Coliform organisms were also detected in the concentrates. Neither virus nor coliforms were detected in 50 ml portions of the unconcentrated water sample. Future studies relating to coliform to virus occurrences in potable water may suggest the desirability of establishing a coliform standard based on 1-liter sample examinations (6). This requirement would increase the base-line sensitivity and could be particularly important for measuring coliform reduction resulting from the application of disinfectants at rates approaching those essential for control of waterborne virus. However, routine bacteriological examinations of potable water presently utilize 50-ml volumes for the multiple tube test or 100-ml portions for the MF technique. Water quality surveillance of streams and estuaries frequently requires smaller test volumes because of significantly higher bacterial densities.

Sample Collecting Procedures

When samples must be hand collected directly from an estuary, river, stream, lake, or reservoir, by wading-in for near-shore samples or from a small boat, the procedure is to grasp the open bottle near its base and plunge it, neck downward below the surface. The bottle should then be turned until the neck points slightly upward, the mouth being directed toward the current. If no current exists, as in a reservoir, a current should be artificially created by pushing the bottle horizontally forward in a direction away from the hand. When sampling from a boat, samples should be obtained from the upstream side of the boat. When sampling from a bridge or large boat, the sterilized sample bottle should be placed in a weighted frame that holds the bottle securely. The sample bottle is then opened and lowered into the water by a small diameter rope or nylon cord without dislodging dirt or other material from the bridge that might fall into the open bottle. As the bottle nears the water surface, the mouth of the bottle is oriented to face upstream by swinging the sampler downstream under the bridge and dropping the unit quickly into the water without excessive slack in the rope. Too much slack in the rope may permit the submerging sample bottle to reach bottom and pick up mud or be broken from impact on submerged rocks. After the bottle is partially filled, the sampler is pulled upstream and out of the water, simulating the scooping motion of sampling by hand. Water samples collected from a well, either by mechanical or hand pumping, must be drawn and wasted for several minutes before the sample is collected. The procedure ensures that water in the well field is sampled and not the standing water in the pump. An additional advantage is that contaminants that might have entered the area of the tap are flushed away.

Potable water samples must be representative of the water quality within a given segment of the distribution network; therefore, taps selected for sample collection must be supplied with water from a service pipe connected directly with the main rather than to a storage tank. The sampling tap must be protected from exterior contamination associated with being too close to the sink bottom or to the ground. Contaminated water or soil from the faucet exterior may enter the bottle during the collecting procedure since it is difficult to place a bottle underneath a low tap without grazing the neck interior against the outside faucet surface. Leaking taps that allow water to flow out from around the stem of the valve handle and down the outside of the faucet or taps in which water tends to run up on the outside of the lip are to be avoided as sampling outlets. Aerator, strainer, and hose attachments on the tap must be removed before sampling. These devices can harbor a significant bacterial population if they are not cleaned routinely or replaced when worn or cracked. Whenever an even stream of water cannot be obtained from taps after such devices are removed, a more suitable tap must be sought. Taps whose water flow is not steady should be avoided because temporary fluctuation in line pressure may cause sheets of microbial growth that are lodged in some pipe section or faucet connection to break loose. The chosen cold water tap should be opened for 2 or 3 minutes or for sufficient time to permit clearing the service line; a smooth-flowing water stream at moderate pressure without splashing should be obtained. Then, without changing the waterflow, which could dislodge some particles in the faucet, sample collection can proceed.

When glass bottles fitted with ground-glass stoppers are used, a string or paper wedge must be inserted between the bottle and closure before sterilization to facilitate easy opening during sample collection. Upon opening the bottle, discard the string or paper wedge without touching the inner portion of either the bottle or stopper. Reinserting this item into the sample bottle after sample collection will increase the risk of water sample contamination.

Regardless of the type of sample bottle closure used, do not lay the bottle cap down or put it in a pocket. Rather, hold the bottle in one hand and the cap in the other, keeping the bottle cap right side up (threads down) and using care not to touch the inside of the cap. Likewise, avoid contaminating the sterile bottle with fingers or permitting the faucet to touch the inside of the bottle. The bottle should not be rinsed or wiped out or blown out by the sample collector's breath before use. Such practices may not only contaminate the bottle but remove the thiosulfate dechlorinating agent. During the filling operation, be careful so splashing drops of water from the ground or sink do not enter into either the bottle or cap. Do not adjust the stream flow while sampling in order to avoid dislodging some particles in the faucet. Fill the bottle to within 1 inch of the bottle top or to the shoulder of the container; cap the bottle immediately. The tap is then turned off.

Flaming Tap Myth

Treating water taps before collecting potable water samples is not necessary if reasonable care is exercised in the choice of sampling tap (clean, free of attachments, and in good repair) and if the water is allowed to flow adequately at a uniform rate before sampling. Alterations in the valve setting to change the flow rate during collection could affect the sample quality adversely. Superficially passing a flame from a match or an alcohol-soaked cotton applicator over the tap a few times may have psychological effect on observers, but it will not have a lethal effect on attached bacteria. The application of intense heat with a blow torch may damage the valve-washer seating or create a fire hazard to combustible materials adjacent to the tap. If successive samples from the same tap continue to contain coliforms, however, the tap should be disinfected with a hypochlorite solution to eliminate external contamination as the source of these organisms (7).

This negative position on a protocol for flaming taps before sample collection is supported by several independent studies. Thomas *et al.*, (8) after a study of 253 samples from farm water supplies, reported that flaming taps before sampling resulted in no significant differences in the multiple tube test (5-tube MPN) for both total coliforms and fecal coliforms, nor in the standard plate counts incubated at 37° or 22°C. They noted that there was a tendency for the bacterial content to be lower, but the trend was not significant and could have occurred by chance. In a second study involving 527 distribution samples collected without tap flaming from the Chicago public water supply, only two samples (or 0.4%) contained coliforms (9). For a third study, water was flushed from taps located in 76 gasoline service stations in Dayton, Ohio, but again, the taps were not flamed or otherwise disinfected (10). The results showed no coliform positive samples from 40 of the 76 stations, and MF coliform counts in excess of 4 per 100 ml occurred in only 4 of the 10,916 samples tested.

Dechlorinating and Chelating Additives

All water samples collected from chlorinated sources must be dechlorinated at time of collection (11,12). Unless residual chlorine is neutralized, the bactericidal activity will continue and decrease the opportunity of detecting any organisms that would indicate a possible contamination in the potable water supply. Before sample bottles are sterilized, a sufficient concentration of sodium thiosulfate is added to each bottle so that after the appropriate volume of water sample is collected, there will be an equivalent 100 mg dechlorinating agent per liter of water. Thus, 4-oz (125 ml) capacity sample bottles require the addition of 0.1 ml (2 drops) of a 10 percent solution of sodium thiosulfate to each bottle, since approximately 100 ml of water will be added during sampling. The use of 6-oz (180 ml) or 8-oz (250 ml) capacity sample bottles requires a proportional increase in the amount of dechlorinating agent added. Excess amounts (greater than 0.4 ml of a 10 percent solution) of sodium thiosulfate should be avoided, since this may encourage bacterial aftergrowth in the standard plate count and, thereby, alter the bacterial concentration in the sample during transit to the laboratory. Sterilization temperatures evaporate the sodium thiosulfate solution to dryness, leaving a thin white film of the dechlorinating agent available to combine with any residual chlorine in the sample. Theoretically, 7.1 mg of sodium thiosulfate will completely dechlorinate 1 mg of chlorine. In practice, this ratio is as low as 2:1 because of the effects on samples of temperature, excess light exposure, and various oxidizing complexes possible in a given water sample.

Inspection of the 10 percent sodium thiosulfate solution, which is used as the dechlorinating agent when preparing the sample bottles, should not reveal the agent to be turbid, either from bacterial growth or from chemical decomposition. Any biological or chemical alteration of the dechlorination agent may adversely affect the detection of some residual coliform population in a marginally chlorinated potable water during sample transit time to the laboratory. Therefore, sterilization of the dechlorinating reagent, preferably prepared in quantities of 50 ml or less, and its subsequent storage in the refrigerator is recommended to reduce the probability of chance contamination.

Chelation of the water sample, as a method maintaining the coliform density during transit, may be desirable in waters naturally containing copper or zinc and in sewage or industrial wastes with high levels of heavy metal ions. These heavy metal ions exert a toxic effect on bacteria and may significantly decrease total and fecal coliform densities during transit periods of 24 hours or more (13,14). Although some of the bactericidal action of copper is prevented by adding 100 mg/l sodium thiosulfate to the sample bottle (15), broader chelation is attainable with ethylenediamine tetraacetic acid (EDTA) at a concentration of 372 mg/l. Thus, it may be desirable to prepare sample bottles with the dechlorinating compound, sodium thiosulfate, and also the chelating agent EDTA. One suggested approach is to prepare a mixed stock solution of the proper concentration of both chemical agents and to add appropriate 0.1- to 0.5-ml quantities, as required, to each sample bottle. Quantities of these chemical agents, added separately or collectively, should not exceed 0.5 ml per bottle since larger volumes will not evaporate to a dry residual during sterilization and liquid residuals may be spilled out through inadvertent inversion of sample bottles during the collection procedure.

SAMPLE IDENTITY—LEGAL CONSIDERATIONS

It is imperative that all laboratory and field personnel recognize the legal aspects associated with collecting either monitoring or surveillance data that could become involved in an enforcement action. In particular, custody of samples must be clearly established from the time samples are taken until the evidence is introduced in court. Sample collectors requested to appear in court must be prepared to state the time and date samples were taken (including assignment of a sample number), identify specific sampling locations, describe field tests performed (chlorine residual, water temperature, and water pH), and validate the sample collector's signature.

These critical requirements make it mandatory for the sample collector to fill out a sample identification form immediately after each sample is taken. A ballpoint pen (waterproof ink) should be used with ample pressure to ensure that all multiple copy forms are legible. Print or write all information clearly. Samples received in the laboratory accompanied by illegible report forms or of questionable identity should not be processed. Notation should be made by the sample collector of any special conditions that may suggest contamination, so that laboratory personnel may prepare proper dilutions to cover the range of possible bacterial concentration. If the sample is part of a resampling program, such as a followup of unsatisfactory potable water results, such information should be noted on the sample identification form. This form must also include a sample bottle identification number that is either permanently marked on each bottle or added with a wax marking pencil or waterproof pen to the side of the bottle. Marking an identification number on the sample bottle closure is not desirable because closures can be inadvertently mixed during sampling or processing in the laboratory.

Laboratory personnel must be responsible for the custody, care, and processing of the sample upon arrival in the laboratory and, therefore, must be prepared to testify to this protective trust in court, if necessary. The laboratory should maintain a logbook to show registration of the sample on receipt from the sample collector, including arrival time and date and initials of the recorder. The laboratory record or worksheet and the sample form submitted with the sample must include information on the procedures performed and the results of the testing and must also be signed and dated by the person performing the tests. Where selected procedures deviate from recommended methods, the laboratory personnel, under cross examination, should be prepared to justify procedural changes by presenting validation data that adequately establish equivalency and sensitivity of the nonstandard tests employed.

SAMPLE TRANSIT FOR STREAM AND MARINE SAMPLES

All water samples, regardless of source, must be examined as soon as possible after collection. Sample transit time is especially critical for stream and marine pollution investigations or for monitoring these stream and marine waters as part of a water quality surveillance program. Because few field studies are in an area adjacent to the laboratory facility, a special courier service must be established to transport all samples to the laboratory within a maximum 6-hour time period. Samples may be transported long distance via air freight in sturdy picnic coolers using prefrozen chemical cold packs to maintain a 4° to 10°C temperature during shipment. This procedure requires coordinated scheduling relating to sample collection, transportation to the airport for shipping, available flights, and transportation from the air terminal to the laboratory for examination. Upon receipt in the laboratory, these samples must be processed within 2 hours to ensure valid data (12,16).

If the sample transit time requirement for a specific field study precludes use of the central laboratory, other alternatives must be sought such as: (a) acceptance of the samples for analyses by an approved laboratory nearer to the study area, (b) examination of samples by an approved water laboratory field kit brought to the field study site, (c) on-site bacteriological testing in a mobile laboratory, or (d) application of the delayed incubation procedure for total coliforms (17) or fecal coliforms (18).

SAMPLE TRANSIT FOR POTABLE WATER SAMPLES

Sample transit time and water temperature variations during transport continue to be a problem for central laboratories that must analyze samples collected from distant water supplies. Transit time should never exceed 48 hours, preferably no more than 30 hours. These potable water samples should be refrigerated whenever a standard plate count is requested or the water is suspected of being contaminated with pathogens. Refrigerated samples held longer than 30 to 48 hours will be subject to unpredictable increasing or decreasing bacterial densities. This problem is amplified with the standard plate count because the general bacterial population undergoes a more rapid change than the coliform density. Insulated sample containers provide some protection against rapid changes in the water sample temperature, and perhaps a thermos-type container that can be sterilized should be considered. Another promising approach is to package the sample in a container engineered to maintain a pre-set temperature in the range of 4° to 10°C for 48 hours.

Changes in bacterial density, in addition to being related to storage time-temperature effects, are influenced by the chemical composition, pH, electrolyte concentration, protein nitrogen, bacterial flora, and other undetermined factors associated with specific water sources. Bacterial nutrients present in a given water may support significant bacterial multiplication during sample transit, particularly at temperatures above 13°C. If storage time is prolonged, the bacterial population may completely exhaust specific nutrients and begin a sharp die away. Thus, samples low in bacterial nutrients and stored for long periods before examination may have undergone a considerable reduction in the original bacterial density because of die off.

Every effort must be made by sample collectors to time mail shipments of drinking water samples with existing mail, truck, bus, or air schedules. Sample collectors should avoid routine sampling on Thursday, Friday, or any work day before a holiday. When samples must be tested at other than regular working hours arrangements must be made with laboratory personnel. If the postal service is unacceptable, shipment by truck, bus, bank clearing house service, or other alternate means of transportation should be investigated. For those water supplies located within 2 hours' driving time of the laboratory, every effort should be made by the sample collector to bring sample collections directly to the laboratory rather than resort to mail service. When samples are to be transported by car, delivery should be done promptly and not postponed to some more convenient time during the next few days. Transporting samples for several hours in the high temperature of a car trunk or on the back seat of the automobile during the summer can drastically alter the bacterial population.

In subtropical and tropical areas, special effort should be made by sample collectors to refrigerate all potable and nonpotable water samples during transit to the laboratory because of the warm water and air temperature. Keeping water samples cool will retard changes in the bacterial density and, thereby, yield laboratory results that are a more meaningful measurement of water quality at time of collection.

Samples shipped by commercial carrier must be adequately protected in suitable shipping cases to avoid breakage or spilling. Where sample collections are made within a reasonable driving radius of the laboratory, sample collectors may use large picnic coolers as sample cases with a 4° to 10°C temperature maintained through use of ice, dry ice, or prefrozen chemical cold packs. If only ice is available as a refrigerant, it may be necessary to modify the sample case by constructing a water-tight center compartment to contain the ice and, thus, avoid any contamination of water sample with melted ice. The laboratory staff should also be authorized to reject any samples submerged in reservoirs of melted ice. This requirement eliminates any doubt concerning the integrity of the collected sample during transit.

Sample processing must be initiated within 2 hours of the arrival time. Where laboratories receive samples throughout the day, the staff should plan to process all morning samples by 11:00 a.m. and samples received during the afternoon after 3:00 p.m. Late sample collection arrivals may necessitate using some staff assistance from other laboratory sections to complete initial processing by close of business. When samples are delivered too late to be examined during the regular work day, serious efforts should be made to authorize personnel for overtime processing. Overnight refrigeration of these late arrivals is a permissible alternative provided processing is done promptly the next morning. Under no circumstances, should samples be stored in the refrigerator during the weekend for processing on the following work day.

UNSATISFACTORY BACTERIOLOGICAL REPORTS

When the bacteriological results from a sample indicate unsatisfactory quality, additional samples from the same location must be examined at daily intervals until two consecutive negative samples are secured. Such special samples should not be included in the monthly total of routine sample examinations required by the Federal Drinking Water Standards. The laboratory should promptly report unsatisfactory sample results to the engineering division and to the water plant operator so that an immediate resampling program is initiated. Slow processing of positive results by the laboratory or engineering records section of the water plant defeats the efforts by the laboratory to maintain a rapid monitoring and warning alert system on public water supplies. When repeat sampling is initiated several days or weeks later, the opportunity is lost to further verify coliform occurrence resulting from short-term water quality deterioration. Because further confirmation through repeat bacteriological sampling is frequently lacking, it might lead to the belief that the positive sample result was a "fluke." To counteract this misinterpretation of the bacteriological results, the laboratory should further verify any positive coliform findings found in samples from public water supplies.

Repeated occurrences of low numbers of coliforms (1 to 10 coliforms per 100 ml) indicate chronic contamination in some portions of the distribution system due to cross-connections, negative pressures during fire emergencies, distribution line deterioration, or inadequate treatment practices. These positive findings should be viewed as an early warning of a break in the protective barrier against pathogen entrance into the potable water supply. Since one waterborne outbreak occurs each month somewhere in the United States (19), the detection of low coliform density levels should be given more consideration.

The recommended course of action is to thoroughly flush sections of the distribution networks supplying water of unsatisfactory bacteriological quality to free them from sediments and chemical deposits, then chlorinate these mains to reduce the bacterial population. Sufficient disinfectant should be added to the finished water as it leaves the water treatment plant to maintain a chlorine residual (preferably ≥ 0.3 mg/l free chlorine) throughout all sections of the distribution network. Booster chlorinators at various points in the system may be needed to maintain this residual. Developing a systematic program for monitoring the chlorine residual and turbidity at representative points throughout the distribution system is also desirable.

On samples that yield MF cultures covered with confluent growth of bacterial colonies, resampling is recommended because the true coliform density may be obscured. In addition to interfering with the development of typical sheen colonies, large densities of nonspecific organisms may inhibit coliform growth. Coliform colonies can occasionally be observed even though there is confluent growth. If four or less coliform colonies are observed under such conditions, a new sample should be requested from the same sampling point since it must be determined whether or not the coliform density exceeds the defined limit. If there are over four coliform colonies, confluent growth or not, action must be taken in compliance with the Federal Drinking Water Standards.

Quantitation of noncoliform colonies from the MF total coliform procedure is of uncertain specific interpretation because M-Endo medium suppression of this nonspecific population approaches 95 to 99 percent. However, these observations of excessive background growth do imply that the general bacterial quality of that treated potable water is below normal attainment by conventional treatment practices. Some of these background organisms may be a factor in creating health problems among the very young, the debilitated, and the aged individuals in a community. In addition, high noncoliform populations in finished water are implicated in suppressing coliform detection in both MF and MPN procedures. Such observations are particularly relevant since the medium utilized to detect coliform organisms in the MF procedure will suppress substantial numbers of the general bacterial population. Therefore, when excessive background growth is observed on the MF total coliform test, the water plant operator should be alerted to submit a special sample for standard plate count examination. Standard plate counts in excess of 500 per 1 ml should justify a recommendation to the water plant operator that the cause of the excessive noncoliform populations be determined and appropriate measures taken to reduce the bacterial density below the suggested health limit. Remedial action may include line flushing to remove accumulating sediments and chemical deposits, determination of dead-end sections, and maintenance of 0.3 mg/l free chlorine residual throughout the distribution lines.

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GUIDELINES ON SAMPLING AND MONITORING RESPONSE

Potable Water Sampling

Representative points selected on the distribution network .... ________
Sampling frequency adequate .... ________

Bathing Water Sampling

Sampling sites reflected water quality within the entire recreational zone .. ________
Sampling frequency related to peak activity periods during the entire bathing season .... ________

Stream Pollution Sampling

Sampling sites within the study area included domestic and industrial effluents, water supply intake, and recreational areas .... ________
Sampling program included a base-line location upstream of the study area ________
Sampling frequency reflected conditions during normal operating and nonoperating hours for industrial plant operations .... ________
Sampling interval of 4 to 6 hours selected for short-term, 7- to 10-day studies ________
Stream and lake monitoring sites reflected overall water quality .... ________

Sample Collection

Care exercised in collecting representative samples .... ________
Sample collected with ample air space in bottle for mixing .... ________
Minimum sample size of 100 ml collected for all types of water samples .. ________
Stream sampling directed into the current and at least 6 inches below surface ________
Well water drawn to waste for several minutes before sampling .... ________
Municipal water tap protected from exterior contamination and free of aerator, strainer, or hose attachment .... ________
Water tap sampled after maintaining a smooth flowing water stream for 2 to 3 minutes to clear service line .... ________
Taps with history of previous contamination disinfected with a hypochlorite solution; flaming tap not necessary .... ________

Dechlorinating and Chelating Additives

Sodium thiosulfate added before bottle sterilization at a concentration of 100 mg per liter for sample dechlorination .... ________
Chelation agent for stream samples added before bottle sterilization at a concentration of 372 mg per liter .... ________

Sample Identification

Sample bottle promptly and completely identified immediately after collection ________
Essential information included: water source, location, time and date of collection, chlorine residual, and sample collector's initials .... ________

Sample Transit Time and Temperature Limits

Transit time for source waters, reservoirs, and natural bathing waters should not exceed 6 hours .... ________
Transit time for potable water samples should not exceed 48 hours, preferably within 30 hours .... ________
Mandatory sample refrigeration provided for all bathing waters, source waters, effluents, and certain drinking waters to be examined for standard plate count or pathogen occurrence .... ________
Optional sample refrigeration provided on routine collections of potable waters for coliform analyses .... ________
Routine sample collections timed to meet existing mail, truck, bus, or air schedules .... ________

Sample collections delivered by car reached the laboratory promptly ..... ________

All samples examined within 2 hours of arrival in the laboratory .... ________

Unsatisfactory Sample Response (Potable Waters)

Unsatisfactory sample defined as three or more positive tubes per MPN test or four or more colonies per 100 ml in MF test .... ________

High priority placed on alerting operator to unsatisfactory potable water results .... ________

Unsatisfactory samples resampled promptly .... ________

Special sample for standard plate count requested when more than 200 noncoliform colonies occur on Endo-type MF media .... ________

Water plant operator alerted to take appropriate control measures when standard plant count exceeded 500 organisms per 1 ml .... ________

CHAPTER III
LABORATORY APPARATUS

Basic laboratory apparatus must be of adequate quality to meet levels of sensitivity, be reliable, and need only minimum service repairs to correct mechanical failure or intolerable fluctuations in some critical characteristic. Long-term laboratory equipment items should be of appropriate capacity to meet the current needs during peak work periods and also have an approximate 50 percent additional reserve capacity for future needs. Choice of equipment to be purchased must relate to those laboratory specifications essential to obtaining reliable test results rather than to nonessential attractive features or to cost alone. Instruction manuals should be carefully read by all technicians for proper understanding of the equipment operation and be available in the laboratory files for reference when service repairs or parts replacement information is required. Technicians should be familiar with basic rules in the operation of delicate instruments, such as the microscope and analytical balance, before approval for their use is granted. All laboratory personnel must have a thorough understanding of operational controls and of properly using drying ovens, glassware washing equipment, and autoclaves in an effort to minimize laboratory accidents related to these equipment items.

AIR INCUBATION REQUIREMENTS

Incubator temperature control is essential to detect organisms of sanitary significance in water. Many bacteria in water are without sanitary significance—they die rapidly in the aquatic environment, come from various unknown sources, are widely distributed in the natural environment, or have no known or suspected association with human or other animal wastes (1-9). Since the major emphasis has been on studies of those species or groups of bacteria derived from contamination by animal wastes, it is necessary to choose an incubation temperature favorable to this specific bacterial segment of the water flora (4,10,11). Thus, the choice of incubation temperature, the length of incubation time, and the necessity for lactose fermentation within these conditions essentially defines an indicator system. Any change in these criteria will redefine the heterogenous collection of bacterial species included in the total coliform bacteria and their sanitary significance.

Unpublished studies performed in our laboratory on a series of polluted Ohio River samples indicate that lowering the incubation temperature below 37°C progressively slows the rate of gas production. In parallel most probable number tests using 20°, 25°, 30°, and 37°C incubation temperatures, the rate and amount of gas production increased as the incubation temperature increased. Recent published data (12) on agar plate counts for *Escherichia coli* isolated from well water, sewage, night-

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soil, and polluted fish indicate that 35°C incubation was the optimum incubation temperature. The next best temperature for *E. coli* recovery was 37°C; other temperatures investigated included 20°, 30° and 44°C.

Reducing the incubator temperature below 35°C increases the problems of interference and false positives associated with noncoliform organisms common to well waters, lakes, and some small streams. False positive results in the multiple tube confirmed test may originate from several sources including anaerobic spore formers of the *Clostridium perfringens* type, spore-bearing aerobic forms related to *Bacillus subtilis*, and the synergistic action of two different organisms neither of which alone can ferment lactose. On the membrane filter, the so-called "paracolon group" of organisms occurs as the most frequent false positive; they produce a sheen reaction as a result of the partial breakdown of lactose. In general, these organisms grow better at temperatures below 35°C. It should be emphasized that any reduction in incubation temperature will change the spectrum of organisms included in the indicator group for water analysis.

Since incubator temperature tolerance must be accurately measured to within ± 0.5°C of 35°C, all thermometers used in this application should include 0.5°C scale divisions as a minimum requirement. Extrapolation of readings on thermometers with only 1°C scale divisions is not sufficiently accurate.

BENCH-TOP INCUBATORS

Bench-top incubators must have sufficient space to accommodate all multiple tube tests or MF cultures during peak work periods. A daily record (preferably a morning and afternoon reading) of the incubator temperature is mandatory in the absence of a recording thermometer. This record should include the date, temperature, and the initials of the person logging the data. Any deviations greater than ± 0.5°C from the 35°C incubator temperature must be corrected by proper thermostat adjustment. Maintaining daily incubator temperature records will also alert laboratory personnel to any gradual temperature changes that may reflect decreased stiffness of a new bi-metallic strip or possible metal fatigue in an older bi-metallic element in the incubator thermostat.

Bench-top incubators should have sufficient insulation to protect the inner chamber from room temperature fluctuations. Generally, water-jacketed incubators are far superior to any others in this respect. Temperature instability may, in part, relate to poor insulation in the non-water-jacketed incubator construction and also to power conservation efforts that include turn-off of air conditioning equipment in the laboratory during evenings and over weekend periods. Periodic decreases in line voltage can also affect optimum operation of the heater elements. Where line voltage droppage is a serious problem, insertion of a powerstat variable transformer between the incubator and power outlet may be necessary to improve supply voltage to the incubator heating elements. Temperature instability may also be caused by locating incubators in or near a window where sunlight or cold air drafts produce large temperature fluctuations within a poorly insulated unit and increase the difficulty of adjusting control settings on the heating elements. Ambient temperature in rooms with temperature controlling equipment should be held within the 65° to 80°F (18° to 27°C) range, and when exceeded, air conditioning of the laboratory may be justified to reduce these incubator temperature fluctuations.

Stratified temperatures and "hot spots" resulting from nonuniform radiation may cause some shelf areas in the incubator to be at higher or lower temperature than the temperature desired. Built-in thermometers generally cannot be assumed to give accurate measurements of the average temperature in the chamber until their accuracy is verified by supplemental measurements made with accurately calibrated thermometers placed on top and bottom shelves. For more accurate reading of chamber temperature, thermometer bulbs must be continuously immersed in water to provide buffering from sudden temperature changes when the incubator door is opened.

Air incubation at 35°C produces a low-humidity environment and adequate broth volume and agar substrates must be retained during long-term incubation. Agar plates incubated 48 hours at 35°C should not have more than a 15 percent weight loss through desiccation. Loss of medium through evaporation causes unfavorable pH changes in broth cultures that can suppress bacterial growth and result in the development of small, poorly differentiated colonies on membrane filter surfaces. Partially submerging a towl in a beaker of water increases humidity in the incubation chamber. The wet towel acts as a wick and produces a large evaporation surface. Slime or mold growth may occur on these towels, so it is necessary to replace them once every other week to prevent such undesirable problems. Some commercial incubators have a built-in water reservoir on the bottom, inside each chamber, to aid in maintaining the humidity at approximately 75 to 85 percent. These reservoirs must be periodically filled with water to replenish water lost through evaporation. A plastic vegetable crisper with a tight fitting lid and a wet towel placed on the bottom may also be used to hold total coliform MF cultures; the filled container is then placed in the 35°C incubator.

INCUBATOR ROOM

Room-size incubators require a more complex environmental controlling system than do bench-top units because of their physical size, the requirements for temperature control within ± 0.5°C of a preselected temperature, and maintenance of 75 to 85 percent relative humidity. The optimum design requires a primary heating source and coarse control to regulate the temperature between 30° and 40°C, and a secondary heating source to generate small inputs of heat that will maintain a temperature ± 0.5°C of the preselected temperature, normally 35°C. These two separate heat generators must be controlled by two different thermostats, with the primary thermostat being designed to cut off the large heat output elements at approximately 32 to 33°C. Residual heat buildup should bring the peak room temperature to about 34 to 35°C at which point the secondary heat source is then activated by another thermostat to establish the final temperature at 35° ± 0.5°C. If the temperature control is improperly adjusted or defective and results in temperature excursions to 40°C, an electronic temperature monitoring system should completely shut down both heating circuits and activate an alarm system to alert personnel of excessive temperature buildup in the unit.

In some instances, incubator rooms are designed that employ only a single heat source and a blower to maintain and distribute heat similar to a forced-air heating system. This approach is more economical but is also subject to problems of excessive temperature variations that frequently exceed the recommended tolerance of ± 0.5°C. Uniform distribution of heat throughout the incubator room is essential. One approach places the primary heating elements on opposite walls near the floor and secondary heating elements on all three walls near the floor. Heat stratification can be prevented by establishing a low-rate air flow through an appropriate air exhaust system. This is best accomplished by constructing intake ports in each wall near the floor close to the heating elements. An exhaust port should be placed in the center of the ceiling with a low speed exhaust fan installed to pull heated air continuously through all portions of the room and then exit it through the exhaust port. High-rate exhaust or blower systems cause increased media evaporation and lower the relative humidity. To maintain the desired relative humidity (between 75 to 85 percent), a controlled humidifying system may be necessary.

Shelf areas in the incubator must conform to 35° ± 0.5°C temperature requirement. In addition to any recording thermometer installed in the incubator room, thermometers, with their bulbs immersed in water, may be placed at several locations in the shelf area. Recording thermometer charts document the extent of temperature cycling and possible instability and drift in the control system. When temperature records reveal a persistent drift or excessive temperature cycling, action must be taken to service the control circuit for pitted, arcing contact points; defective by-pass condenser; or metal fatigue in bi-metallic strips.

ELEVATED TEMPERATURE INCUBATION REQUIREMENTS

Various procedures recommended for selective recovery of *Salmonella*, rapid (7 hour) detection of fecal coliforms, and the *Standard Methods'* fecal coliform procedure require incubation ranging from 41.5° to 44.5°C depending on the specific test chosen. Precise temperature control is essential since temperatures lower than those recommended will permit the growth of many nonspecific organisms, and temperatures higher, decrease the recovery of the desired pathogen or indicator group. Once the test is prepared, the inoculated media should be brought to the desired temperature within 10 to 15 minutes and held precisely within the recommended range. For these reasons, incubation in a water bath or in a solid heat sink incubator (such as aluminum) is desirable because precise temperature control in these systems is more easily attained than it is in air incubators.

Accurate temperature measurements are essential for elevated temperature tests. A continuous temperature recorder sensitive to 0.2°C changes should be used for a permanent record. In addition, an accurate thermometer must be immersed in the water bath to spot check the precision of the recorder tracings once each day. If recorder tracings are inaccurate, the ink pen should be adjusted so that the temperature tracings agree with comparative readings of the immersed thermometer. If a recording thermometer is not used to monitor water bath temperatures, a daily record of temperature readings from an immersed thermometer or digital electronic thermometer must be made.

Since water bath or heat sink block temperature tolerance must be accurately measured to within ± 0.2° of 44.5°C, all thermometers used in this application should include 0.1°C scale divisions as a minimum requirement. Extrapolation of readings on thermometers with only 1° or 0.5°C scale divisions is not sufficiently accurate.

Circulating water baths or heat sink block incubators may not have to be kept turned-on during nonuse periods of 72 hours or longer provided the laboratory has established, through adequate data, that the desired stable temperature can be achieved prior to time of use. Noncirculating water baths must be left on at all times since stability in these units, at the recommended temperature tolerance of ± 0.2°C, is marginal.

WATER BATH MAINTENANCE

Large bench-top water baths with gabled covers can effectively maintain a temperature of 44.5°C within ± 0.5°C. Temperature measurements in these noncirculating water baths may reveal that some are capable of temperature control within ± 0.2°C; others exhibit a slightly greater deviation. These latter water baths can be brought to within ± 0.2°C temperature tolerances by adding a low speed stirring motor to create a gentle circulation of water to prevent heat stratification. Coarse temperature control and inadequate heat diffuser bottom plates may create more severe temperature control problems.

Stainless-steel or plastic-coated baskets and racks should be used in water baths to avoid problems of metal corrosion. Heavy deposits of rust from baskets and tube racks made of ferrous material accumulating as sediment in the water bath may act as a heat insulator and must be removed. Adding a rust inhibitor to the water bath will reduce rust formation. A water bath rust inhibitor may be prepared by dissolving 2 grams of potassium or sodium dichromate and 0.5 gram of sodium carbonate or 1.0 gram of sodium bicarbonate each in a little water and adding to the water bath separately because a violent heat reaction occurs if both compounds are added to water at the same time.

Water baths that develop a slime or fungal growth or that become contaminated by accidental culture spills may be disinfected by adding 1 ml of a 10 percent Roccal solution (or equivalent organic quaternary ammonium compound) per gallon of bath water or by adding liquid laundry bleach at the rate of 1 tablespoon per 20 gallons of water (13). After a 24-hour contact period, the water should then be drained from the bath, and the bath should be flushed and refilled with distilled water.

MODIFYING SEROLOGY WATER BATHS

Large-size serology water baths with top covers may be converted to incubators with the more exacting temperature requirements so necessary for fecal coliform incubation (14). An electronic control relay, thermoregulator, water pump, and water intake diffusing pipe are needed. The switching contacts of the electronic control relay must be rated at 1,650 watts or more to match the wattage demands of the heating elements and be wired for 115 volts or 230 volts, depending upon the heating circuit requirements. Response sensitivity of this electronic control relay should be 250 microamps to match the characteristics of the thermoregulator. Temperature range of the thermoregulator should be from 10° to 105°C (50° to 220°F), with a sensitivity to changes of ± 0.01°C (0.02°F).

Provisions for water circulation require external connection of a simple centrifuge pump powered by a ¼-horsepower electric motor. Tygon tubing connects the water circulating pump to intake and discharge ports on one side of the water bath chamber. The input port is connected to a diffusing pipe made of ⅝-inch-diameter polyvinyl chloride plastic pipe (or other noncorrosive materials) with diffusion holes ⅛ inch in diameter on two sides at 2-inch intervals. For an alternative water circulation system, submersible pumps may prove adequate if a gentle circulating water current can be created. When water circulation is combined with an adequate temperature controlling circuit, water temperature can be held within a ± 0.1°C variation.

DRY HEAT STERILIZATION

Commercial-type ovens used to sterilize glassware items should be checked to verify that the 170°C (338°F) sterilization temperature is reached and is maintained within ± 10°C temperature change for a 2-hour period. This is of particular concern where kitchen-appliance-type ovens are adapted to laboratory use; dial control calibrations of these ovens are frequently inaccurate and must be calibrated by an accurate thermometer.

Since both time and temperature are interrelated in sterilization, all appliance-type and laboratory-designed ovens should preferably include an accurately calibrated recording thermometer for more precise timing of the sterilization process. As a minimum requirement, however, a long-stem thermometer of known accuracy in the range of 160° to 180°C should be inserted through a center ceiling port, with the bulb inserted into a cylinder (e.g., 25-ml graduated) filled with fine sand and positioned on the center shelf in the sterilization chamber. Immersion of the bulb in a small container of sand will better simulate average temperature conditions in pipette or petri dish containers and in thick-walled glass sample bottles. The sand acts as a buffer against sudden temperature changes when the oven door is opened and permits more accurate calibration of the oven sterilization temperature following the recommended 2-hour sterilization period. Additionally, the sand prevents rapid temperature fluctuations that cause the mercury column to suddenly contract over a large section of the capillary and thereby increase the chance of introducing air-space separations in the mercury and loss of temperature-measuring accuracy.

A long-stemmed thermometer is necessary—the bulb is located near the center of the sterilization chamber and the upper portion of the scale, in the range of 150° to 200°C, is visible outside the oven for temperature readings while the oven doors are closed. To protect the thermometer from breakage, the top of the oven should not be used as a storage area. Likewise, care should be exercised during loading of the oven so that the bulb portion of the thermometer in the sterilizing chamber is not similarly broken. Repeated breakage of thermometer bulbs may be a result of operator carelessness or overloading the ovens with items to be sterilized. The oven must be of sufficient size to prevent crowding of the interior.

AUTOCLAVES

Autoclaves are essential for preparing many sterile items including bacteriological media, sample bottles, and membrane filter equipment, as well as for decontaminating test culture discards. This equipment should be of adequate capacity to prevent crowding the interior, which would result in ineffectual sterilization of some items in baskets or trays packed tightly together.

The chamber of the autoclave should be equipped with an accurate thermometer with bulb properly located in the exhaust line so that it registers the minimum temperature in the sterilizing chamber. Pressure gage readings generally correlate with sterilization temperatures, but must not be used as a sterilization guide because certain variables can distort this relationship, such as incomplete exhaustion of all air in the chamber. Therefore, the sterilization period should correlate, primarily, with that time when the necessary chamber temperature plateau is achieved.

The use of the recording thermometer built into the automatic autoclave provides essential information related to rate of initial temperature acceleration, maximum temperature achieved, constancy of sterilization temperature during predetermined time period, rapidity of exhaust, and total exposure time during the complete sterilization cycle. This record is important in evaluating effects on sterilization of various carbohydrate media and in detecting unsatisfactory changes in automatic cycles and impending equipment failure. Such records, developed from the appropriate daily or weekly charts recommended for the specific recorder, should be dated and type of material autoclaved identified for each specific sterilization cycle, then stored for possible reference use over a minimum of 2 years. Retention of these records is necessary in the event such evidence is needed in future laboratory evaluation studies or in disputes on health risks related to decontamination procedures for microbiologically hazardous material discards.

Vertical autoclaves and household pressure cookers may be used in emergency service if equipped with pressure gages and thermometers with bulbs positioned 1 inch above the water level. However, they are not to be considered the equivalent of the general purpose steam sterilizer recommended for permanent laboratory facilities. Their small size is inadequate for large-volume work loads, and they can be difficult to regulate.

Labeling tapes with heat-sensitive color changing inks, heat-sensitive crayons, or other materials that change color or physical state are useful for autoclave control procedures. Heat resistant spore suspensions of *Bacillus stearothermophilus* in culture medium that are killed only when exposed to 121°C for 15 minutes (15,16) also provide a positive control on autoclave procedures when the spores are incorporated into culture media. These sterilization indicators should be used each time the autoclave is operated. Placing sterilization indicators in the central area of a large load of materials to be sterilized will monitor heat penetration to the most protected items.

Steam for the autoclave may be supplied from a saturated steam line, or from gas, or from an electrically heated steam generator incorporated in the equipment item. Autoclaves connected to a building supply of saturated steam usually reach sterilization temperature more quickly than autoclaves that require an accessory steam generator. Poorly regulated steam pressure may come from electric steam generators or autoclave chambers that heat slowly because portions of the heating elements fail or because the units are of insufficient wattage to generate an adequate steam supply. Similar difficulties may occur with gas-fired steam generators that are poorly adjusted or that are equipped with jets not designed for use with the commercial gas available in the community.

Autoclaves must receive periodic inspection and preventive maintenance. Drains in the autoclave chamber can become clogged from spills and boil-over of materials during sterilization. The drain should have a screen cover to retain coagulated wastes that block liquids. Gaskets around the door crystallize and crack with age, and temperature and pressure dials can have broken protective dial faces and damaged instrument pointers. Some laboratories have been found using ancient autoclaves that are no longer manufactured. Replacement parts for these deteriorating units are not always available, and substitute parts may or may not be adequate for the specific unit. In the event an older unit or a unit of unknown manufacture cannot be properly brought up to acceptable safety standards, a new autoclave should be purchased. Serious accidents have occurred from malfunctioning autoclaves exploding and from ruptured steam fittings.

LABORATORY THERMOMETERS

The accuracy of all thermometers routinely used to monitor temperatures in incubators, water baths, hot-air sterilizing ovens, and autoclaves must be verified by comparing their readings with readings of a National Bureau of Standards (NBS) certified thermometer or one of equivalent accuracy. Preferably, every laboratory should own an NBS certified thermometer set because of the importance of exact temperature control.

Certified thermometers are expensive items that must be carefully protected during use or while in storage to avoid breakage or separation of the mercury column. Each certified unit has its plot of accuracy enclosed with the NBS certificate of acceptance; this information is critical for use in establishing precise temperature measurements and calibration of routine thermometers under test. If the certification sheet with a plot of calibration corrections has been lost, it will be necessary to request a reissue of the certification plot for that specific thermometer from the NBS or to submit a set of selected thermometers to that organization for calibration at temperatures commonly used in the laboratory. When this latter service is requested, selected calibration points should include temperatures most commonly used, such as 5°C, 20°C, 35°C, 44.5°C, 121°C, and 170°C. Thermometer stem length is important in a calibration thermometer so that divisions of 0.1°C can be easily read. However, there is one disadvantage to long-stem thermometers in that any irregularities in the mercury column bore will be reflected in drift from precise measurements. Therefore, when temperatures of a wide range of values must be calibrated, it may be more desirable to have several calibrated thermometers of limited overall range rather than one unit to cover a wide range of values.

Occasionally, in every laboratory, thermometers break and must be replaced with new units. Replacement thermometers should be verified for accuracy within the minimum and maximum range of intended use since thermometer accuracy is not uniform over the entire stem length. All "in-use" thermometers should be rechecked periodically for development of hairline breaks in mercury columns that decrease measuring accuracy. Air space separations can be eliminated by carefully submersing the thermometer in high- and low-temperature water baths, taking care that the maximum temperature does not exceed the thermometer range. Thermometers with poorly legible graduation marks should be discarded.

pH METER

Laboratory pH measurements must be made with an electronic instrument capable of direct readings within ± 0.1 pH units. Models with tube circuits are subject to occasional service problems related to tube failure. The problems range from poor electronic emission, gas build-up, and internal noise to heater element failures. Preventive maintenance should include a periodic check of tube characteristics with the use of a mutual conductance tube tester if available; otherwise, new tube substitution should be made when the pH meter response becomes questionable.

Newer models of pH meters are built with solid state devices that are more reliable than tube circuits but that require professional electronic repair service if they become faulty. The problem frequently relates to defective electrolytic condensers or resistors that change values and that may, in turn, affect the operational characteristics of transistors and result in transistor breakdown.

Electrodes may also become defective at the thin-walled tip and cause erratic performance; therefore, a spare replacement electrode should always be available. The calomel electrode must be maintained with a full reservoir of saturated potassium chloride solution at all times so that pH standardization and subsequent meter readings do not become erratic. When not in use, electrode tips should be immersed in a small beaker of distilled water to prevent them from becoming dry and caked with potassium chloride crystals. Loss of potassium chloride in the electrode chamber can be controlled during storage by inserting the rubber plug at the filling port and using the rubber cap over the electrode tip to retard the slow bleeding of saturated potassium chloride through the fiber element in the tip. Of course, during periods of operation, it will be necessary to remove both the rubber cap and plug from the electrode to permit test solution contact and to equalize liquid pressures. The same general precautions described above apply to pH meters using combination electrodes.

If erratic meter readings are observed when the hand is held near the electrode, check the electrical grounding system for the instrument. Poor grounding or the lack of instrument grounding will cause instability because of a hand capacitance effect on the instrument. Placing pH meters on metal table tops can also cause a similar inductive capacitance interference. Although placing the instrument on a thick nonmetal stand above the metal surface may help, it is generally more desirable to move the instrument to another table with a nonmetal working surface.

Colorimetric pH methods are not acceptable in the bacteriological laboratory because it is impossible to make pH determinations of strongly colored solutions such as brilliant green lactose bile (BGLB) broth or M-Endo MF medium.

BALANCE

Media preparation requires a balance capable of weighing several hundred grams or more. For this purpose, each laboratory should have a torsion type balance or trip pan balance with a sensitivity of better than 2 grams per 150 gram load. Such balances may have all or part of the weights built into the system or weights may be added separately in a counterbalance pan. Balance weights should be kept in a protective box when not in use, free from chemical spills, and handled only by the forceps supplied to ensure continued weighing accuracy through years of usage.

Care should be taken to avoid sudden jolts or jarring during weighing procedures to protect the delicate knife edge on the balance point. Always lock the balance before moving it to some new operating position, use a cover where possible to protect the instrument from dust, and avoid spilling dehydrated media on the mechanism. Many of the dehydrated bacteriological media are very hydroscopic and can cause erratic dampening of the balance point during zero balance or weighing operations.

An analytical balance with 1-mg sensitivity at 10-gram load is used for weighing media additives, reagents, dyes, etc., which are added in amounts less than 2 grams. This type of sensitive balance must be protected from vibrations, dust, and wind currents generated by heating/cooling ventilation systems, or areas of busy laboratory traffic. Because this is a very delicate instrument, actual use of the analytical balance should be limited to staff members who have demonstrated a thorough knowledge of its proper operation and care. An annual preventive maintenance program of balance adjustment, cleaning, and repair by a qualified instrumentation-service organization should be established by every laboratory.

MICROSCOPE AND LIGHT SOURCE

MF colonies are best counted using 10 × to 15 × magnification. A binocular, wide-field dissecting microscope is recommended as the best optical system. Use of a reading lens for this purpose is ineffective because the low magnification power does endanger properly detecting small colonies and defining numbers of differentiated colonies occurring in clumps of confluent growth. Examination of MF total coliform cultures with the unaided eye is not recommended because small sheen colonies or those with a faint or atypical sheen may go undetected.

The golden metallic luster of coliform colonies, the blue colonies of fecal coliforms, the red colonies of fecal streptococci, and various other differentiated colonies on other selective media are best observed with diffused daylight developed from cool-white fluorescent lamps, with the light source adjusted to an angle of 60° to 80° above the MF culture (17). Low-angle lighting must be used on MF cultures growing on nonspecific growth media without indicator systems. On these general growth-type media, nonpigmented colonies appear gray-white and require shadow-contrasting to aid their detection on the gray-white MF surface.

A fluorescent light source consisting of two 4-watt daylight tubes mounted on a flexible arm attached to a heavy cast base is recommended for MF colony illumination. High-intensity incandescent illumination commonly used with oil immersion microscopes or the low-intensity incandescent light produced by an illuminating flashlight magnifier are not adequate for colony sheen observations on Endo-type media. Confusion over colony appearance may lead to errors in differentiated colony counts.

COLONY COUNTER

Accurate and standardized counting of colonies on pour plates requires a special device with adequate back lighting. Some colonies are difficult to detect when viewed by top lighting but are readily seen when illuminated by a uniform intensity, transmitted light. A large-diameter magnifier of approximately 2 power is necessary not only to see the smallest colonies but also to distinguish pin-point colonies from particles of dissolved medium or precipitated matter in the agar. A Wolfhügel guide plate or other grid plate of crisscross lines is essential for guiding the eye in scanning an agar plate culture and for ensuring systematic coverage without inducing overlapping colony counts. These requirements are met by the Quebec colony counter, preferably the dark-field model that reveals colonies of bacteria clearly against a dark background.

INOCULATING EQUIPMENT

Transfer of bacteriological growth from broth, agar, and MF cultures to some secondary medium or to a microscope slide requires the use of several different types of inoculating aids. In the multiple tube confirmed test, culture transfers from positive presumptive tubes are usually performed with an American Wire Gauge (AWG) number 22 to 24 wire loop made of chromel, nichrome, tungsten, or platinum-iridium. The single-loop diameter should be 4 mm or greater, preferably between 6 and 7 mm, to provide adequate transfer of broth without accidental spillage of contents. The standard loop used to obtain 0.001-ml sample volumes in milk examinations is too small (being only 1.45 mm in diameter) for the transfer of growth from positive presumptive tubes to the confirmatory broth. The wire shank on all transfer loops should be between 7 and 8 cm long to reach the culture broth without contaminating the tube with the inoculating loop holder.

Wire loops made of approximately 85 percent platinum and 15 percent iridium (or rhodium) alloy with an AWG number 22 thickness are preferred in many laboratories because of their fast cooling after flame sterilization. Nichrome wire is less expensive and is stiffer but does not cool as quickly. When ordering nichrome wire, it is advisable to choose AWG number 24 thickness because of the greater stiffness of this alloy compared with platinum-rhodium.

For laboratories using the multiple tube procedures for fecal streptococcus detection, a triple loop should be available for confirming positive azide dextrose broth cultures to ethyl violet azide medium. This confirmatory medium requires a larger inoculum because of the growth suppressive agents incorporated in its formulation to reduce false positive reactions. The triple loop can be constructed from any of the previous alloys by twisting a single length of wire into a chain of three individual loops at the free end of the wire shank.

Alternatives to the flame-sterilized wire loop for culture transfer include single-service transfer loops of aluminum or stainless steel (18). Quantities of these transfer loops may be placed in a modified, short-length, stainless-steel pipette container or in a large-diameter, glass test tube with a protective, metal-foil cover and sterilized either by dry heat or steam. After using a single transfer loop, it should be placed in a beaker containing a suitable germicide. Occasionally stainless-steel loops become tarnished from exposure to concentrated germicides or from charred materials accumulated in the dry heat sterilization process. Tarnish may be removed from stainless-steel loops by placing them in a glass cylinder and *very carefully* adding boiling sulfuric acid. After 5 or 10 minutes in the cleaning solution, the acid solution is very carefully drained into another acid-resistant container. Slowly add tap water to the cylinder of loops to rinse the residual acid from the stainless steel loops. Repeat the rinse several times, and then remove the transfer loops. The black film remaining on the loops can now be easily removed by polishing with sandsoap or household scouring powder.

Disposable, single-service, hardwood applicator sticks that have been sterilized by dry heat may also be used for transferring broth cultures (19). Steam sterilization must not be used because wood distillate products may be generated that are toxic to bacteria in the transfer procedure. Hardwood applicators (1/12 to 1/8 inches in diameter) must be long enough to reach the bottom of the culture tube with at least 1 inch extending out of the tube for manipulation. Single-service hardwood applicators used for culture transfer are a convenience in field and mobile laboratories. Flame sterilization is not required, and there is adequate inoculum pick-up from the presumptive tube to inoculate a BGLB broth tube and EC broth tube without recharging the applicator. These sterile applicators may also be used to transfer growth from a coliform colony on M-Endo MF to lauryl tryptose broth in the initial verification step.

The use of glass straws (Pasteur pipettes) is not a recommended culture transfer practice because of the excessive quantity of inoculum introduced into the BGLB broth. This heavy inoculum may introduce sufficient densities of noncoliform flora that suppressive agents (brilliant green dye and bile salts) in the medium may be inadequate. The net result could be a failure to eliminate false positive fermentation reactions from the confirmation of lactose gas positive cultures in the presumptive test.

Inoculating needles are commonly used to transfer growth from MF cultures, agar plates, or pure culture slants for further purification, biochemical tests, or serological slide agglutination procedures. The inoculating needle may be made from any of the aforementioned alloys and should be sufficiently long to avoid contaminating the lip of a culture tube during transfer. Inoculating needles used for streaking plates should have a smooth tip to prevent tearing the agar surface. If a microwelder or similar heat source is available, the needle tip of some metal alloys may be made into a smooth ball by momentary application of heat. Specify an alloy wire gauge size that will provide the necessary stiffness for making agar stab inoculations or for picking subsurface colonies for transfer to other media.

MEMBRANE FILTRATION (MF) UNITS

Filtration assemblies for the MF procedure consist of two parts: the funnel and a funnel receptacle. The receptacle that supports the MF on a porous metal screen or glass frit is generally mounted in a suction flask or a special drain system with a No. 8 rubber stopper. A funnel is clamped or twist-locked to the receptacle during filtration and directs the flow of water over the effective filtration area of the MF. Reusable filtration assemblies may be constructed of autoclavable plastic, borosilicate glass, spun stainless steel, or metal plate materials. Funnels manufactured of stainless steel are less subject to corrosion and are very durable under field use. Glass and plastic funnels are graduated for direct measuring, and unless subjected to frequent breakage, they cost less.

Filtration assemblies should not leak during the filtering procedure. Worn lock wheels on the funnel-locking ring assembly of metal units or improper seating of the membrane on glass filter units frequently cause leaking. Inspection of the narrow neck of the funnel sometimes reveals worn areas in the metal plating that expose the brass base material. Since brass is toxic to bacteria, such worn funnels should not be used. All surfaces of the filtration assemblies in contact with the water sample before its passage through the MF should be uniformly smooth and free from corrugations, seams, or other surface irregularities that may retain bacteria.

It is recommended that the funnel portions of each filtration unit be washed at least once each week in a mild detergent solution to prevent the accumulation of a dirt film or water hardness spots on the funnel walls. Grease and soil deposits can become areas that block the free-flowing funnel rinsing action required after each filtration. As an added precaution, these units may be coated with a silicone preparation such as "Desicote." This hydrophobic coating prevents the metal or glass from being wetted and minimizes sample retention on surfaces of the funnel. Silicone coatings on filtration equipment will withstand repeated autoclave sterilization. When equipment is used daily, the silicone coating should be renewed monthly.

FORCEPS

Sterile forceps (alcohol flamed) are necessary in the manipulation of MF's, both for placing them on the filtration apparatus and for transferring filters to broth saturated pad or agar media. Forceps should be constructed with smooth, spade-shaped ends similar to forceps used in stamp collecting. Sharply pointed forceps or forceps with knurled inner surfaces on the ends should not be used because of the risk of puncturing or tearing the membrane. Use a metal file to modify such forceps for a more blunt, rounded end with smooth inner surfaces. Forceps with a slightly curved tip for better manipulation around the curvature of the culture dish are acceptable.

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GUIDELINES ON LABORATORY APPARATUS

Incubator

Manufacturer________________________ Model ________________

Sufficiently sized for daily work load .... ________

Uniform temperature maintained in all parts (± 0.5°C) .... ________

Thermometer accurately calibrated, with bulb immersed in water on top and bottom shelves .... ________

Temperature recorded daily or recording thermometer sensitive to ± 0.5°C change used .... ________

Incubator not subjected to excessive room temperatures—variations beyond a range of 65° to 80°F (18° to 27°C) .... ________

Incubator Room (Optional)

Manufacturer________________________ Model ________________

Well insulated, equipped with properly distributed heating and humidifying units for optimum environmental control .... ________

Shelf areas used for incubation conformed to 35°C ± 0.5°C temperature requirement .... ________

Thermometers accurately calibrated with bulb immersed in water .... ________

Temperature at selected areas recorded daily or recording thermometer sensitive to ± 0.5°C changes used .... ________

Water Bath

Manufacturer________________________ Model ________________

Sufficiently sized for fecal coliform tests .... ________

Uniform temperature of 44.5°C ± 0.2°C maintained .... ________

Thermometer accurately calibrated, immersed in water bath .... ________

Temperature recorded daily or recording thermometer sensitive to ± 0.2°C changes used .... ________

Hot-Air Sterilizing Oven

Manufacturer________________________ Model ________________

Sufficiently sized to prevent crowding of interior .... ________

Construction ensured a stable sterilizing temperature .... ________

Thermometer accurately calibrated in range of 160° to 180°C or equipped with recording thermometer .... ________

Autoclave

Manufacturer________________________ Model ________________

Sufficiently sized to prevent crowding of interior .... ________

Construction provided uniform temperature up to and including 121°C .... ________

Thermometer accurately calibrated with bulb properly located to register minimal temperature within chamber .... ________

Pressure gage and operational safety valve provided .... ________

Steam source provided from saturated steam line or from gas or electrically heated steam generator .... ________

Sterilization temperature reached in 30 minutes .... ________

If pressure cooker used, provided with a pressure gage and thermometer with bulb 1 inch above water level .... ________

Thermometers

Accuracy checked against a thermometer certified by National Bureau of Standards or one of equivalent accuracy .... ________

Liquid column had no discontinuous sections; graduation marks legible ... ________

pH Meter, Electronic

Manufacturer________________________________ Model ____________________
Accuracy calibrated to 0.1 pH units .... ________

Balance

For general media preparations, Type ______________ balance used,
with 2-gram sensitivity at 150-gram load .... ________
For weighing quantities less than 2 gram, Type ______________
analytical balance used, with 1 mg sensitivity at 10 gram load .... ________
Appropriate weights of good quality provided for each balance .... ________

Microscope and Lamp

The preferred binocular, wide field, type used; 10 to 15 diameters
magnification for MF colony counts, Type ______________ ...... ________
Fluorescent light source provided .... ________

Colony Count

Preferred Quebec colony counter, dark-field model for standard
plate counts used .... ________

Inoculating Equipment

Wire loop of 22- or 24-gage chromel, nichrome, or platinum-iridium,
sterilized by flame, used .... ________
Single-service transfer loops of aluminum or stainless steel, presterilized
by dry heat or steam, used .... ________
Disposable single service hardwood applicators, presterilized by
dry heat only, used .... ________

Membrane Filtration Units

Manufacturer________________________________ Model ____________________
Leak proof during filtration .... ________
Metal plating not worn to expose base metal .... ________

Forceps

The preferred round tip without corrugations used .... ________
Forceps alcohol flamed for use in MF procedure .... ________

CHAPTER IV
GLASSWARE, METAL UTENSILS, AND PLASTIC ITEMS

Glassware items used during the daily operations of a laboratory are repeatedly subjected to a variety of corrosive materials in testing procedures, high temperature during sterilization, vigorous cleaning schedules, and careless handling; all of these speed glassware to ultimate discard and replacement. Technological improvements have introduced disposable hard-glass (borosilicate) items, an extensive choice of plastic substitutes, and some stainless-steel vessels for use in the once exclusive domain of laboratory glassware. However, substitution with disposable or reusable plastic items must be fully evaluated in terms of labor costs, possible reassignment of some nontechnical preparation room personnel to other responsibilities, suitability of reuse plastic items, and a continued availability of selected stock items from the supplier. Plastic materials used in a bacteriological laboratory must be free from toxic residual lubricants used in the molding process, exhibit clarity, have accurate calibration marks for volume measurements, and withstand repeated autoclaving if the items are to be reused.

MEDIA PREPARATION UTENSILS

Utensils made of borosilicate glass or other suitable noncorrosive material, such as stainless steel or enamel, are recommended for use in preparing media. If enamelware is used, it must be free of chips in the procelain-like glaze that expose the base metal to corrosion and to interaction with media preparations. Utensils made of aluminum, copper, or zinc alloys should not be used because these metals also react with media solutions and introduce metal ions that are toxic to bacterial growth.

Utensils for media preparation must be thoroughly cleaned to prevent carry-over of foreign residues or dried medium. When metal utensils are used, care must be taken to clean crevices around handle rivets or other attachments that might harbor caked deposits of previously prepared media. Magnetic stirrers that are inserted in large glass Erlenmeyers to aid in a more rapid solution of the dehydrated media must also be thoroughly cleaned.

SAMPLE BOTTLE SPECIFICATIONS

Wide-mouth sample bottles should be used for all water collections because they permit sample collection with less chance of accidental contamination at the water tap or other outlet port. Glass sample bottles should be made of borosilicate or other noncorrosive glass, preferably with metal or plastic screw-cap closures that incorporate a nontoxic, leakproof liner. Screw caps require no protective dust cover since the design of the closure affords adequate protection during normal storage and sampling procedures.

New plastic screw caps on glass sample bottles should be checked for bacterial toxicity by using a modification of the distilled-water suitability test. A minimum of 10 sterile plastic caps should be soaked in 100 ml sterile distilled water for 24 hours at room temperature. This water is then examined for toxic residuals with the use of the distilled-water suitability test. The plastic caps that have toxic residuals, as reflected by the results of the rinse water examination, should not be used until free of these toxic properties. In most instances, these unsatisfactory plastic caps can be detoxified of phenol residuals after six successive autoclavings in repeated changes of distilled water.

Ground-glass stopper closures are acceptable if they are covered before sterilization with a metal foil, rubberized cloth, or heavy impermeable paper that extends from the cap to the shoulder area of the bottle. Paper or cloth covers must be held in place by string or tape that can withstand sterilization temperatures. Foil covers are preferred and can be held in place by pressing the foil around the narrow portion of the bottle neck. This requirement protects the inner portion of the cap and bottle top from contamination during the storing, handling, and transporting to or from sample collections. Always keep the cover over the ground-glass cap while handling, and once the sample is collected, replace the cap with its cover pressed over the bottle.

The rising cost of shipping samples by mail has prompted replacing glass sample bottles with plastic containers that can withstand autoclave sterilization for 15 minutes at 121°C. These plastic sample bottles are available with wide mouth openings and screw-cap closures. Some difficulty with autoclavable plastic bottles may be related to the purchase of polyethylene bottles that are not as rigid a plastic as is the polypropylene or polycarbonate type. In either case, plastic bottles should not have the screw caps tightly closed during sterilization, so that changes in air pressure and elevated temperatures will not cause some of these bottles to exhibit a partial or complete collapse of the side walls. Sample bottles made of linear polyethylene with a polypropylene screw closure should not be used because leakage can occur when samples are held at refrigeration temperatures. Apparently the difference in the coefficient of expansion rate for these two different plastic materials is the source of this problem. Therefore, specifications for plastic sample bottles should require the bottle and screw closure to be of the same autoclavable plastic material.

Plastic bottles for bacteriological samples offer advantages of low cost, light weight, and resistance to breakage. However, they must not contain toxic substances or organic matter that originates from the plasticizer or mold release agents. The presence of adverse substances can be determined by using the procedure previously described for plastic screw caps.

Sterile plastic bags ("Whirl-Pak" type) may be useful for limited sample collections involving unchlorinated waters. Problems associated with preventing contamination, with leakage, and with aseptically adding a dechlorinating agent restricts using these plastic bags when collecting chlorinated water. Attempts have been made to inject the dechlorinating agent into each bag and cover the puncture with water-proof tape. However, leakage may occur at the puncture point if the tape is not completely water resistant and firmly attached.

Stream samples may be shipped in these plastic bags provided the filled bags are properly folded for closure and then reinforced with freezer tape for a positive seal to prevent leakage. Following these precautions, water samples in plastic bags can be transported either in boxes or heavy paper envelopes ("Jiffy" or equivalent) without leakage. Tests for residual toxicity indicate that after prolonged storage (over several months) toxic substances can leach into the water samples, presumably because of leaching of plasticizers. For the time limits established for transporting bacteriological samples, however, this low toxicity level is insignificant.

PIPETS

Pipets may be of any convenient size, provided they deliver the required volume quickly and accurately within a 2.5 percent tolerance. Pipets that are graduated to the tip should be discarded if the tip is broken since the measurement of a sample will not meet the required calibration tolerances. Tip-delivery, 10-ml pipets with narrow openings are undesirable because of the slow flow of measured 10-ml portions. The tips of such pipets should not be cut to increase the flow rate because of the reduced accuracy in measured volumes.

Graduation marks must be legible and permanently bonded to the glass. Pipets made of glass formulations other than borosilicate are often more susceptable to etching during cleaning procedures. Even pipets constructed of borosilicate glass will become frosted if allowed to stand for extended periods (overnight) in a caustic detergent solution. Pipets that become badly etched should be discarded because of poor visibility of the fluid meniscus. Disposable plastic pipets must not only be sterile but also must meet the required tolerance for calibration accuracy and legibility and be free of toxic residues introduced during manufacture and commercial sterilization.

A cotton plug may be inserted into the mouth end of each pipet as a safety measure to prevent the technician from accidentally ingesting caustics, volatile solvents, or other dangerous agents including pathogens in sewage and industrial wastes. The use of cotton-plugged pipets is optional, but when employed, the cotton plug should not be so tight fitting that it obstructs drawing quantities into the measured length of the pipet nor so loose as to fall out of the mouth end before or during use. Optionally, a rubber bulb or mechanical pipetting aid may be used.

PIPET CONTAINERS

Metal boxes or cans used for sterilization and storage of sterile pipets should not be constructed of copper since this metal is very toxic to bacteria. The high temperatures required for sterilization of pipets can oxidize copper particles and cause them to flake off and be transferred into the culture media when these pipets are used to deliver measured sample portions. Therefore, it is recommended that all copper pipet containers be replaced with stainless-steel containers that resist heat deterioration. Aluminum pipet containers are acceptable but generally less durable than stainless-steel units. Also, individual pipets may be wrapped in good quality paper that will resist charring and brittleness caused by sterilization temperatures. Because of the large number of pipets used, wrapping pipets in paper for sterilization and storage is impractical in most laboratories. Metal boxes or cans permit easy access to the pipets as well as convenient storage for large numbers of pipets during high-volume work periods.

A pad of nonabsorbent cotton, glass wool, Teflon, or silicone rubber placed in the bottom of the pipet can is occasionally used as a protective cushion to reduce breakage of pipet tips. Although this practice affords some measure of protection for tip-delivery pipets, the cotton or glass-wool padding may become a source of debris, which can then become trapped on the MF surface during sample pipetting. Repeated exposure of these protective pads to high sterilization temperatures causes the padding to become brittle and small pieces are easily picked-up on the tips of pipets stored in these cans. When such protective pads are used, it is necessary to replace them whenever deterioration is observed.

PETRI DISHES

Petri dishes are essential laboratory items for standard plate count determinations, MF cultures, and streak plate isolation of bacterial cultures. Where these culture dishes are used for pour plates and pure culture isolations, the size is usually 100 mm × 15 mm. Special studies requiring the examination of more than 2- to 3-ml volumes by the pour plate procedure necessitate a larger volume of agar to solidify the increased sample volume and, therefore, a larger diameter Petri dish. Since the MF procedure is standardized on 47-mm diameter membranes, the tight fitting 50- × 12-mm Petri dish is generally used for this technique, although other sizes can be used if desired.

Although glass Petri dishes have long been used in the laboratory, the use of disposable plastic Petri dishes is increasing because of their overall lower cost and the elimination of washing and sterilization procedures and risk of breakage. Regardless of the material (glass or plastic), these culture dishes must be completely transparent for optimum visibility of colonies, have flat bottoms to eliminate uneven dispersion of suspended bacterial cells in the pour plate technique, and be free from bubbles and scratches that impair observation of deep colonies. Plastic dishes with tight fitting covers, in contrast to the loose-fitting standard Petri dish covers, are preferred for MF cultures because they retard evaporation loss from broth or agar media and they help maintain a humid atmosphere in the culture dish.

PETRI DISH CONTAINERS

Metal containers (stainless steel or aluminum) are essential for properly sterilizing and storing glass Petri dishes since they have loose fitting tops that could allow dust to contaminate the sterile inner portions of the dish. Copper containers should not be used since copper readily oxidizes or flakes off after repeated exposure to dry-heat sterilization temperatures. Such particles are toxic and might be introduced into the Petri dishes and medium additions. Since Petri dish containers for sterilizing the 60-mm-size glass-type Petri dish are not readily available as a manufactured item, heavy metal foil can be used to roll wrap approximately 6 to 10 dishes for sterilization. The heavy metal foil, folded at top and bottom, holds firmly without additional support. Char-resistant paper sacks or wrapping paper may also be used to prepare Petri dishes for sterilization.

Plastic, disposable Petri dishes are packaged, presterilized, in plastic bags as a protection against airborne contamination. It is desirable to open one plastic pack at a time to minimize chance contamination. Some disposable Petri dishes used for MF cultures are packaged in small cardboard boxes, which are also useful as protective storage containers for the dishes in the laboratory. In fact, if the small plastic dishes are sterilized for reuse (see subsection on Plastic Culture Dish Reuse in Chapter V), they may be stored in the same boxes, or in plastic boxes with tight fitting covers commonly used for refrigerated food storage.

CULTURE TUBES AND CLOSURES

Cultures tubes are used for a variety of purposes, including multiple tube procedures, biochemcial tests for bacterial identification, and stock culture collections. These tubes must be of a sufficient size to contain the culture medium, as well as the sample portions employed, without being overly full. When culture tubes are too small, cultures are more subject to contamination and may become the source of contamination during transfer by spilling, splashing, or generating aerosols.

Since observation of gas production is essential to multiple tube procedures and various biochemical test reactions, an inverted vial (fermentation or gas vial) must be inserted into culture tubes being prepared for fermentation tests. The length and bore of the inverted vial should be related to the culture tube size and volume of medium. The medium volume should be sufficient to ensure complete filling of the inverted vial during sterilization and to partially submerge the fermentation tube at least half-way. The diameter of the inner tube should not be less than 40 percent of the diameter of the culture tube with which it will be used. A common practice is to use 16- or 18-mm × 150-mm culture tubes with 10- × 75-mm fermentation tubes for biochemical fermentation tests and multiple tube procedures involving sample portions of 1-ml or less. Where 10-ml sample portions are needed, larger culture tubes of 25-mm × 150- or 200-mm size must be used. However, the same size (10- × 75-mm) fermentation tube inserts are permissible. The use of smaller fermentation vial inserts make early observation of gas bubbles more uncertain.

Snug-fitting stainless-steel or plastic caps (2), or loose fitting aluminum caps, are the recommended closures for culture tubes used in the multiple tube procedure. Since these closures cover the lip and upper inch of the culture tube, flaming the tube opening is not necessary when pipetting or transferring a culture with an inoculating loop or needle. Because metal caps are more durable than plastic or cotton plugs, they are more economical over a long period or for indefinite reuse.

Although nonabsorbent cotton plugs may be used as tube closures, much time is required to prepare them satisfactorily. Cotton plugs should extend 20 to 30 mm into the tube and approximately 30 mm out from the tube opening for proper handling during sample pipetting or culture transfer. When cotton plugs are used, the culture-tube capacity should be large enough to adequately contain the desired medium and sample volumes without wetting the plug during sterilization or sample processing. Once a cotton plug is wet, it loses its effectiveness as a barrier. Since these plugs do not protect the upper edges of the tube opening, this area must be flamed to reduce the risk of contamination. Because polyurethane foam test tube plugs may inhibit microbial growth by release of volatile fatty amines to the growth medium during autoclaving (3), do not use any polymeric material unless toxicity tests demonstrate it to be inert.

Culture tubes with screw-cap closures are preferred for use when preparing media for biochemical tests and agar slants for stock culture collections. With the tighter fitting screw closure, broth and agar preparations can be stored for longer time periods without excessive media loss through evaporation. Of several sizes of screw-cap culture tubes available, the 16- × 150-mm size is frequently employed in biochemical tests and the 16- × 125-mm size is preferred for agar slants used in stock culture maintenance.

All culture tubes must be made of borosilicate glass or other corrosion resistant glass. Whenever these tubes become frosted from the corrosive action of improper cleaning or chemical reagents or become excessively scratched from use to the point that visibility is impaired, they must be discarded. Disposable culture tubes are generally made of soda-lime glass (soft glass); these are not recommended for bacteriological use because of interaction of glass and media during storage. Another disadvantage of soda-lime glass is its susceptibility to etching during routine glassware cleaning. These limitations mitigate against the use of most single-service culture tubes in the bacteriological laboratory.

DILUTION BOTTLES OR TUBES

Examination of bacterial populations by the multiple tube test, MF procedure, and pour plate technique requires preparation of accurate sample dilutions. Dilution water blanks are prepared in either screw-cap culture tubes containing 9 ml of diluent for 1:10 dilutions or in dilution bottles that have a capacity for 99 ml diluent. This latter approach is more common since it permits both 1:10 and 1:100 dilutions to be prepared from the same suspension.

These glassware items must be made of borosilicate or other corrosion resistant glass with a graduation level for 9 ml (tube) or 99 ml (bottle) permanently marked on the glass wall. This mark will assist the bacteriologist to detect occasional dilution blanks that may contain improper volumes of dilution water, thereby necessitating their rejection for use in preparing serial dilutions. Because a source of screw-cap culture tubes with a permanent 9-ml mark may not be readily available from most commercial suppliers, a quantity of appropriate borosilicate glass culture tubes must be calibrated at the 9-ml level with a diamond marking pencil or other glass marking tool. These special culture tubes, like all other dilution bottles, should be restricted to preparation of dilution water blanks and not be mixed with other glassware collections for general laboratory use.

Closures for dilution bottles and tubes must prevent leakage of the contents during vigorous shaking or mixing to obtain uniform suspensions. Therefore, closures must be ground-glass stoppers, rubber stoppers (Escher type), or screw caps. Cotton plugs or metal or plastic caps are completely unsuitable. Each dilution bottle employing either a ground-glass stopper or rubber stopper must be covered before sterilization with metal foil, rubberized cloth, or an impermeable paper cap to minimize contamination of the lip of the bottle while in storage and during hand manipulaiton of the closure during use. Screw-cap closures are preferred because they afford a protective shield over bottle openings and eliminate the need for additional cover.

New plastic screw-cap closures should be checked for bacterial toxicity since some lots have been found to have a phenol-type residual due to toxic substances carried over from the molding process. Six successive autoclavings in repeated changes of distilled water are needed to remove the toxic material.

The toxicity of screw caps to bacteria can be checked by soaking 10 new screw caps in 100 ml sterile distilled water for 24 hours at room temperature, then performing a suitability test on the distilled water (rinse water) used to soak these plastic caps. Those caps that have toxic residuals (reflected by poor bacterial recovery in the suitability test), should be put through the leaching procedure previously described and then retested for toxicity.

Dilution bottles that become chipped or cracked around the neck or have defective liners should be discarded. In addition, bottles with mismatched ground-glass stoppers should also be discarded. In all of these cases, leaky bottles may result in hazardous aerosols that can contaminate the laboratory and expose personnel to an unnecessary risk.

MEMBRANE FILTER QUALITY FOR MICROBIOLOGY

Commercial brands of MF's may vary in performance as a result of manufacturing technology, materials, and degree of quality control exercised. For microbiological applications, there must be a complete retention of organisms on the surface of a nontoxic, inert matrix that permits a continuous contact with nutrients from a medium held in a substrate below the membrane. These basic conditions place demanding requirements on the quality of every membrane used in the laboratory. Basic difficulties encountered with MF's generally relate to pore distribution, nonwetting filter areas, grid-line ink restriction, membrane materials, sterilization practices, and poor storage characteristics that cause increased filter brittleness and surface warping (4-15).

MF pores should be uniformly distributed and have a diameter of 0.45 micron (± 0.02 micron) for routine bacteriological techniques. Pores of some commercial lots of MF's have been found to be so small in some areas of the filter that serious local reduction in the flow rate occurs. The filter should be free of visible nonporous areas that prevent the diffusion of nutrients to the upper surface of the membrane. Any bacterial cells entrapped on such surfaces will not develop into visible colonies because of lack of nutrients. When M-Endo is used in a test of diffusibility, nonwetting areas on the filter will remain white and dry. Such observations should not be confused with air bubbles, which can be removed by reseating the membrane over the medium-saturated pad or agar base. At the other extreme, pores larger than 0.70 micron will not retain organisms associated with indicator groups. For complete bacterial separation from liquids, MF porosity of 0.22 micron is required to ensure retention of the smallest bacteria through physical impingement or electrostatic entrapment.

The ink used to imprint the grid system on the MF should be nontoxic to all bacteria cultivated on the filter surface (6). Some inks have been found to be bacteriostatic or bactericidal. Such effects can be recognized through restrictive colony development adjacent to the imprinted lines. These growth restrictions may not only be caused by inhibition from toxic inks, but also from thick ink imprints that "wall-in" grid squares and hydrophobic inks that prevent nutrient diffusion to sites in the ink imprint. As an additional characteristic, inks selected for grid imprinting should not "bleed" across the membrane surface after a 24-hour contact with any medium normally used at 44.5°C incubation. Heavy imprinting of the grid system can also result in a network of "canal-like" indentations that frequently become filled with confluent growth.

The physical structure of the MF material should be such that it provides an optimum retention of bacteria on or near the upper surface with little migration to areas within the pore matrix. Where subsurface penetration occurs, growth should not be obscured from visual recognition during the colony-counting procedure.

Chemical composition of MF's has largely been limited to polymerized cellulose esters, since MF technology initially developed in this direction. Conventional media designed for selective recovery of bacterial indicator groups or pathogens using agar pour plates, streak plates, or broth cultures had to be redesigned to compensate for the physical-chemical properties characteristic of nitrocellulose materials (5, 15, 16). For example, the selective adsorption of dyes excluded the use of acid to neutral dyes as indicator systems and necessitated the use of increased amounts of brilliant green as a suppressive agent in Kaufmann's brilliant green agar to obtain the desired suppression of some of the unwanted bacterial population. Similarly, when more refined peptones such as tryptone, polypeptone, and proteose peptone No. 3 were added to MF media, they were found to be superior to crude peptones added to the original media. The result has been the creation of a family of media designed specifically for use with nitrocellulose MF products. With these experiences in mind, manufacturers should be careful about revising the Goetz MF process. Changes involve the risk that recommended media may suddenly become less sensitive or less selective. Some compounds introduced to the MF may improve flexibility or flow rate, or stabilize porosity. However, these substances should not become a source of fermentable carbohydrates that cause false colony differentiation, create pH shifts in the indicator systems, are selectively toxic for specific organisms, or adversely depress the selective action of differential media by providing the bacteria with a highly nutritive organic compound. In essence, MF's should remain inert to bacterial reaction and unchanged in those physical-chemical characteristics that affect media selectivity and sensitivity.

Since the bacteriologist is working with aqueous solutions, a test for extractables must be based on water as the solvent. Testing procedures for aqueous extractables should include examination of extractables from the MF's.

Extractable Test

Soak MF's in a double distilled water or high quality water produced in a reverse osmosis system, for 24 hours at 50°C. Decant this water and perform a water suitability test (see current *Standard Methods*) using the original high-quality water as a control. Nutritive extractables will produce a significant growth response in this test whereas toxic extractables will result in substantial reduction of bacterial recovery compared with that of the control.

Membrane Response

In a fecal coliform test using a natural sample, compare the presoaked membranes from the extractables test with MF's not presoaked and of the same lot. Choose a sample such as stormwater runoff, farm pond, or sewage lagoon to obtain a water flora with a high nonfecal coliform flora that might appear as a background growth in this test. Check MF cultures for significant increases in density of nonfecal coliforms (that could interfere with fecal coliform detection), blue color development of fecal coliform, and fecal coliform recovery. Nutritive additions could affect differential characteristics of the test to produce excessive background growth. These additives may also affect medium pH, which in turn could be responsible for poor indicator color. Of course, toxic additives will cause reduced fecal coliform recovery.

Membrane Filter Reuse

In an emergency, MF's may be reused several times, provided these filters are used only in the same medium cultivations. For reuse, discarded filters are washed in three successive changes of gently boiling water. Damaged membranes are removed, and the remaining filters are boiled in 3 percent hydrochloric acid for several minutes. The acid solution is then discarded and the MF's are washed in at least three changes of gently boiling distilled water. A trace of bromocresol purple pH indicator solution and sufficient sodium bicarbonate to neutralize any residual acidity is added to the final rinse water. Following a 5-minute boil in this final rinse water, the MF's are ready for reuse (18). The pink color on MF's acquired from use of an Endo-type medium may be removed by presoaking in a 10 percent sodium sulfite solution (19) before proceeding to the acid and neutralized rinsing procedure.

ABSORBENT PADS

Bacteria retained on the MF surface may receive nutrients from a broth-saturated absorbent pad or from an agar-based medium. When a liquid culture medium is preferred, the absorbent pad substrate material must be of high quality paper fibers, uniformly absorbent, and free of sulfites, acids, or other substances that could inhibit bacterial growth. When the quality of the absorbent pads is suspected of contributing to erratic bacterial growth, the following tests are recommended:

Absorption Capacity

Place an absorbent pad in a culture dish. Slowly add 1.8 ml of M-Endo broth to the pad and allow to stand 10 minutes. Repeat this procedure using a second pad in another culture dish and add 2.2 ml of M-Endo broth. After 10 minutes, the first pad should have absorbed all 1.8 ml of broth whereas the second culture dish should have some excess liquid remaining that was not absorbed. When the absorbent pads saturated with medium are observed visually, they should not exhibit nonabsorbent spots.

Total Acidity

Soak 10 sterile absorbent pads in 100 ml of distilled water overnight. Test the leachate water for total acidity; acidity should be less than 1 mg when the leachate water is titrated to the phenolphthalein endpoint pH 8.3 with the use of 0.02 sodium hydroxide. Also, include a control test for the pH of distilled water used in this test of total acidity in absorbent pads.

Toxic Residuals

Soak 10 sterile absorbent pads in 100 ml of distilled water for 24 hours at 35°C. Perform a suitability test on the distilled water (rinse water) used to leach any soluble substances in the pads. Any test lots of absorbent pads that are found to contain toxic substances that will leach out, as demonstrated by the suitability test, should not be used unless pretreated to improve quality. The pretreatment process for removing toxic materials, such as bleaching agents, consists of soaking pads in distilled water held at 121°C for 15 minutes in the autoclave, decanting the rinse water, and repackaging pads in large Petri dishes for sterilization at 121°C for 15 minutes, using rapid exhaust to quick-dry the pads (17).

The alternative approach is to prepare all MF broths with the addition of 1.5 percent agar. Note, however, that these agar preparations must be carefully added to culture dishes so as to create a smooth, moist surface free of pock marks caused by foam and rapid mixing of air bubbles in the liquid agar preparation. Agar preparations may be used immediately or stored in a cool, dark place and used any time within 30 days, provided no dehydration occurs.

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GUIDELINES ON GLASSWARE, METAL UTENSILS, AND PLASTIC ITEMS

Media Preparation Utensils

Borosilicate glass .... ________
Stainless steel .... ________
Utensils clean and free from foreign residues or dried medium .... ________

Sample Bottles

Wide-mouth, glass or plastic bottles used: ________ capacity .. ________
Sample bottle closure:
Glass-stoppered bottles protected with metal foil, rubberized cloth, or kraft type paper .... ________
Metal or plastic screw-cap, nontoxic and with leakproof liner .... ________
Plastic bottles used that can withstand sterilization (15 minutes at 121°C) .. ________
Sterile plastic bags ("Whirl-Pak" type) available for unchlorinated stream samples .... ________

Pipets

Brand ________ Type ________
Calibration error less than 2.5 percent .... ________
Tips unbroken, graduation distinctly marked .... ________
Delivered accurately and quickly .... ________
Mouth end plugged with cotton (optional) .... ________

Pipets Containers

Boxed in aluminum or stainless steel .... ________
Paper wrapping of good quality sulfite paper (optional) .... ________
Disposable pipet sterile packs unopened till needed .... ________

Petri Dishes

Brand ________ Type ________
100 × 15 mm dishes used for pour plates .... ________
50 × 12 mm tight-fitting dishes (preferred type) used for MF cultures .... ________
Clear, flat bottom, free from bubbles and scratches .... ________

Petri Dish Containers

Aluminum or stainless-steel cans with covers, or heavy metal foil, or char-resistant paper sacks or wrappings .... ________
Disposable plastic dishes protected from direct contamination .... ________

Culture Tubes and Closures

Sufficiently sized for total volume of medium and sample portions .... ________
Borosilicate glass or other corrosive resistant glass .... ________
Metal or plastic caps; plastic (nontoxic) or cotton plugs .... ________

Dilution Bottles or Tubes

Borosilicate or other corrosive resistant glass .... ________
Dilution bottles free of chips and cracks around the neck .... ________
Graduation level indelibly marked on side of bottle or tube .... ________
Screw cap with leak-proof liner .... ________
Plastic screw caps tested for freedom from toxic substances on sterilization ________
If rubber stoppers (Escher type) or ground-glass stoppers used, the required metal foil, rubber cloth, or paper cap provided .... ________

Membrane Filters

Manufacturer________________________________ Type______________________
Full bacterial retention, satisfactory filtration speed .... ________
Stable in use, free of nutritive or toxic additives .... ________
Grid marked with nontoxic ink .... ________

Membrane Filter Reuse

Used only in emergency .... ________
Used only on same medium .... ________
Used only after proper cleaning .... ________

Absorbent Pads

Manufacturer________________________________ Type______________________
Filter paper free from growth inhibitory substances .... ________
Uniform thickness permitting 1.8 to 2.2 ml medium absorption .... ________

Filter Funnels

Manufacturer________________________________ Type______________________
Leak during use .... ________
Badly scratched, scored, or corroded .... ________
Properly sterilized .... ________

CHAPTER V
LABORATORY MATERIALS PREPARATION

Careful preparation of media, thorough cleaning of glassware, and proper sterilization of media and equipment are hallmarks of a properly functioning laboratory support unit. Quality control of these processes involves maintenance of records on media pH and sterilization temperature, toxic residual test on detergent used in the glassware cleaning procedure, suitability test on distilled water, and protective storage of reagents, sterile media, and clean glassware items. Without careful attention to these services, the quality of laboratory data will be compromised both in test sensitivity and reproducibility.

CLEANING GLASSWARE

The high rate of glassware turnover in large laboratory operations requires prompt recycling of dirty, used glassware through the cleaning procedure to produce chemically clean items for reuse in the laboratory with minimum breakage loss. The cleaning operations in large laboratories involve processing 10,000 or more pieces of glassware daily, including test tubes, flasks, beakers, sample bottles, graduated cylinders, pipets, Petri dishes, filter funnels, and some bulky specialized items such as carboys.

Mechanical glassware washing equipment can rapidly clean a large volume and variety of laboratory items without the need for a large staff for this essential service. Mechanical washers must be equipped with high-pressure, directional jet streams to break up and remove stubborn deposits such as microbiological growth films, autoclaved proteins, agar, sediments, sludges, chemicals, and wax markings. Washers must be easy to load (preferably front loading at waist height for operator convenience) and have accessory racks that can accommodate a variety of commonly used laboratory glassware items. For operator protection, the washer should have a built-in safety switch that automatically shuts the washer off if the door is opened during operation. Wash, drain, and rinse cycles must have separate adjustments for cycle time and cycle programming and be automatic in selected sequences for use of 160°F (71.1°C) detergent wash water, a clean water rinse at 180°F (82.2°C) and a final rinse in distilled water or equivalent. The best cleaning cycle is one that will produce sparkling clean glassware, free from acidity, alkalinity, and toxic residues that could suppress the growth of microorganisms (1).

The bacteriologist is responsible for demonstrating that washed glassware is free of toxic or inhibitory residues resulting from the detergent used in the washing procedure. The test for detergent suitability should be performed with glass Petri dishes as follows:

1. Wash and rinse some glass Petri dishes according to the usual procedure.

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2. Rinse another group with six successive rinses of distilled water.
3. Wash some with detergent wash water (in-use concentration) and drain dry without rinsing.
4. Sterilize all three groups.
5. Using standard plate count agar, pour duplicate or triplicate plates with the same water sample (to yield 20 to 60 colonies per plate) on Petri dish sets cleaned as in step 1, 2, and 3. Incubate the pour plates at 35°C for 48 hours.
6. If the unrinsed plates (step 3) have a lower bacterial count (15 percent or more) than the well-rinsed plates (step 2), the detergent contains bacterio-static substances and another detergent should be selected.
7. If the plates in group 1 have bacterial counts lower (15 percent or more) than the well-rinsed plates (step 2), toxic residues remain on glassware from routine washing procedures, and a nontoxic detergent must be substituted for the one in use and a longer rinse cycle must be established.

Since many laboratories now use plastic Petri dishes exclusively, testing the suitability of detergent to clean glassware items should be modified for application to culture tubes or dilution bottles as follows:

1. Wash and rinse some culture tubes or dilution bottles in the usual cleaning procedure.
2. Rinse another group with six successive rinses of distilled water.
3. Wash some of these culture tubes or dilution bottles with detergent wash water (in-use concentration), and drain dry without rinsing.
4. Dispense 20 ml of nutrient broth in each set of tubes or dilution bottles, and autoclave at 121°C for 15 minutes.
5. Inoculate each tube or dilution bottle with 1 ml of the same water sample used for the standard plate count determination.
6. Incubate all tubes or dilution-bottle cultures at 35°C for 24 hours.
7. Prepare appropriate dilutions of these cultures (10^{-4}; 10^{-5}; 10^{-6};10^{-7} ml) and pour duplicate or triplicate plates using standard plate count agar; incubate for 48 hours at 35°C.
8. If plates from unrinsed tubes or bottles (step 3) show a lower bacterial count (15 percent or more) than the well-rinsed tubes (step 2), the detergent contains bacteriostatic substances and another detergent should be selected.
9. If plates from tubes or bottles (step 1) washed and rinsed in usual manner show a lower bacterial count (15 percent or more) than the well-rinsed tubes or bottles, toxic residues remain on glassware. These results indicate a nontoxic detergent must be substituted for the one in use and a longer rinse cycle must be established.

In laboratories where mechanical glassware washers are not available or if glassware items are not cleaned properly by mechanical methods, hand washing must be employed. Hand washing requires the use of detergent formulas specifically developed for laboratory use rather than the mild compounds commonly used in home dishwashing. Wash water must be hot (160° to 170°F) and items should be vigorously brushed with appropriately sized laboratory brushes to ensure removal of normal film deposits and residual deposits of all dried materials. These deposits must be removed from fermentation tubes, culture flasks, and sample bottles to ensure that these residuals do not contribute contaminating material to new media or to cultures when glassware items are returned to laboratory use. The washing procedure must be followed by a hot water rinse and a final distilled water rinse to ensure complete removal of the washing detergent and any chemical deposits.

All glassware items must be inspected after air drying for sparkling clarity. Fogging and etching of glassware may be caused by corrosive reagents, culture byproducts, aging of glass material, excessive concentrations of alkaline detergents or abrasions from handling and from hand cleaning with worn test tube brushes. Glass fogging may also result from adding detergent to dirty tubes before sterilizing the contaminated discards or by reusing soft glass items such as "Dura-Glass" or disposable flint glass. Film deposits may result from using a wash water cooler than 160° to 170°F. Washing glassware in wash water below 160° to 170°F will not remove various residues associated with industrial waste, dairy, and food samples.

Sample bottles used to collect potable water should not be excluded from an adequate wash in detergent solution. In one laboratory, such sample bottles were given only a hand rinse in demineralized water before adding the dechlorinating agent and sterilizing with hot air. This practice resulted in the gradual buildup of a residual deposit inside the bottom third of each bottle that eventually hardened through repeated high-temperature dry-heat sterilizations and left a permanent dark-brown stain. Although this stained material in the bottle may not have contributed toxic material to the water sample since it was essentially baked into the glass, it did present an unsightly appearance that would give the general public an impression that potable water samples were collected in undesirable containers.

Glassware items that have a persistent dirt film may be cleaned by soaking in an organic acid detergent such as "Kleenz-Air" or "Nu-Kleen" (Klenzade Products, Beloit, Wisconsin) or other equivalent products. A suitable method utilizes a 10 percent solution of one of these cleaning aids, including soaking overnight at 140°F (60°C) if possible, rinsing, and then cleaning by the regular automatic washing procedure. For a preliminary cleaning of grease, fat, and oil from pipets and other glassware, the use of 50 percent ethanol, followed by a tap water rinse and a final rinse in 95 percent ethanol, might prove useful. Experience with the problem of removing stubborn dirt films from glassware indicates that the organic detergent procedure will remove most of these deposits; those that remain are permanently etched in the glass. These permanently stained glassware items should be discarded.

STERILIZATION PROCEDURES

Various sterilization procedures are employed in the laboratory. The choice of method depends on the stability of media, reagents, or materials to be sterilized. Common sterilization practices involve: moist heat (steam or hot water), dry heat, complete incineration, gas sterilization, filtration, or UV radiation. In some cases, intermittent exposure to flowing steam is used to sterilize materials that cannot be autoclaved, gas sterilized, or filtered.

Media and Reagents

Tube media and specified reagents should be autoclaved at 121°C, preferably for 10 to 12 minutes and not exceeding 15 minutes, unless there are specific directions for another temperature or time. Tubes must be packed loosely in baskets for uniform heating and cooling. Timing of the sterilization period starts when the autoclave reaches 121°C. The maximum elapsed time for exposing carbohydrate broths to any heat (from the time the autoclave door is closed until the medium is removed from the autoclave) is 45 minutes; this time should be kept to the absolute minimum necessary to achieve sterility. Excessive exposure of sugars, especially lactose, to heat may result in hydrolysis and/or carmelization that, in turn, will give false-positive reactions with some noncoliform bacteria. Additionally, in media containing carbohydrates, amino acids, and peptides, other products may be formed that are toxic to bacteria.

Media preparations for MF procedures are generally not subjected to autoclaving for several reasons, including destruction of sodium sulfite in Endo-type media, instability of some suppressive agents in other media formulations, and the small volumes of media needed on a daily basis. Some exposure to heat is necessary, however, to ensure complete dissolution of all active media ingredients. Therefore, MF media should be heated just to the initial boiling point and then cooled to room temperature. This heating procedure is best accomplished by placing the flask of medium in a boiling-water bath for 5 minutes. As medium temperatures reach an approximate peak of 97°C during this period, the preparation becomes essentially sanitized. Direct heating of the medium on a hot plate is not desirable since the medium must be frequently swirled and immediately removed when the boiling point is reached. Subjecting a flask of medium to the intense pin-point heat of a Bunsen flame is also not recommended because of the rapid temperature increase to boiling and the probability of medium destruction.

Reagents added to various media including antibiotics, sugar solutions, and stock buffer water are frequently sterilized by membrane filtration because these solutions may be heat sensitive or become chemically contaminated when exposed to live steam. In the filter sterilization procedure, only clear solutions can be processed because particulate matter will rapidly clog the MF pores. Bacteria and larger microorganisms will be separated from liquids by 0.22-micron-size MF's. Sterile filtrates should be collected in appropriately sized, sterile, screw-capped or ground-glass-stoppered containers and stored at 4° to 10°C.

Membrane Filters and Absorbent Pads

Sterilization of the MF is essential to all applications involving filtration of liquids for bacterial removal or for use in bacterial cultivation. Before the development of the Goetz MF process, membrane filters were sterilized in the laboratory by gently boiling them in distilled water for 20 minutes and repeating the procedure a second time with fresh distilled water. This procedure served the double purpose of sterilizing the membrane and of extracting any residual toxic substances. In retrospect, the continued use of this leaching and sterilization procedure would have avoided many of the variations in MF performance now evident. However, the procedure does take more time to execute and is a recognized inconvenience in busy laboratories examining 50 to 200 samples per day.

MF's may be purchased in resealable kraft envelopes also containing 10 absorbent pads. These units are packaged for autoclaving or are presterilized. If not presterilized, packets of MF's and absorbent pads must be sterilized before use by autoclaving at 121°C for 10 minutes. Immediately following the 10-minute period, the autoclave should be rapidly exhausted to atmospheric pressure and the membranes promptly removed from the autoclave to minimize heat exposure. Excessive exposure to sterilization temperatures may cause MF pores to seal, creating uneven flow-through during filtration, or cause membranes to become brittle and distorted. This problem is also aggravated by sterilization of MF stocks held in storage for periods beyond 18 months. Rapidly exhausting the autoclave also reduces the amount of water condensate retained by the absorbent pads. Moisture retained in the absorbent pads not only reduces their absorption capacity but also alters the final medium concentration.

Commercial presterilization of MF's may be done by autoclaving, gamma radiation, or exposure to ethylene oxide. A comparative study of presterilized membranes has suggested that there are significant increases in bacterial recovery rates for steam-sterilized MF's compared with those sterilized with ethylene oxide (2). Therefore, for those laboratories that are using supplies of membranes sterilized with ethylene oxide, it may be desirable to submit several packs to steam sterilization (121°C for 10 minutes with rapid steam exhaust) to further flush out latent toxicities. These membranes should then be compared with other membranes from the same lot of ethylene-oxide-treated membranes in a pure culture recovery experiment. Some residual toxic effect might possibly persist from ethylene oxide reaction products.

Despite manufacturing claims to the contrary, nitrocellulose MF's do undergo some change in their physical characteristics during storage. Upon aging, MF's may lose their flexibility and break apart at pressure points created during manipulation. During filtration, surface warping often occurs and a complete contact with the medium substrate becomes impossible. The solution to this problem is not to stockpile MF supplies beyond the estimated need for a 12-month period.

MF Filtration Equipment

The equipment used for the MF funnel and membrane holder can be constructed from stainless steel, glass, polycarbonate, or polypropylene plastic materials. This equipment should be sterilized by autoclaving at 121°C for 15 minutes, after having been cleaned and wrapped in kraft paper to maintain sterility during laboratory storage. To sterilize at the laboratory bench between filtrations, expose the filter funnel, with cleaned surfaces, and the membrane holder to UV light for 2 minutes (3). Take appropriate measures, however, to screen stray UV light from the operator's eyes and skin and from MF cultures being processed adjacent to the sterilization unit. Operator protection from skin burns is best provided by placing the UV unit in an enclosed cabinet or bench drawer so the unit can be activated only after the cabinet or drawer is closed. If an open cabinet is used, coat the interior walls with a black paint to reduce the incidence of light bounce out of the UV unit.

Although the average life of the UV lamp may be 5000 hours, the practical lamp life depends primarily on characteristics of the individual lamp and the number of times it is used. Therefore, maintaining a log on the number of hours of operation and relating this to manufacturer's recommended time limit for germicidal capabilities is of questionable value. Bacteriological tests of the germicidal effectiveness of a given UV source should be conducted periodically (4).

A suitable measurement of the effectiveness of UV exposure consists of preparing a coliform pure culture suspension in buffered dilution water so that 1 ml of diluent contains approximately 200 to 250 organisms and exposing the suspension to UV light for measured time periods. Pipet 1 ml of this suspension into each of two sterile Petri dishes. The suspension in one open Petri dish is exposed to the UV radiation for 2 minutes and the control suspension in the other Petri dish is exposed only to the lighting in the laboratory for 2 minutes. After the 2-minute exposure, pour plates of these two cultures, using standard plate count agar, are prepared and incubated at 35°C for 48 hours. Comparative colony counts on plates from the UV-exposed and unexposed suspensions must indicate that UV exposure is producing a 99 percent kill of the bacterial suspension. This bacteriological procedure should be carried out at regular intervals so that a general pattern of lamp life under normal laboratory use can be established. Once the life expectancy of the lamp is established, a reasonable time pattern for routine replacement can be determined.

Although glass funnels can be sterilized by immersing in a boiling-water bath for not less than 5 minutes, this is a hazardous practice that can lead to serious burns as a result of accidental splashes and spills. An additional disadvantage occurs in humidity buildup from flowing steam vapor escaping into the surrounding working area.

Dry heat sterilization (170°C for 1 hour) can be used for glass filter assemblies if the rubber stopper on the receptacle is removed before heat exposure. This approach is not acceptable for sterilizing either metal or plastic units, however. Neoprene or nylon lock wheels on metal funnels undergo rapid deterioration and plastic filter assemblies become distorted due to the high temperature and long-time exposure with the dry heat sterilization procedure.

Sample Bottle Sterilization

The sterilization procedure for sample bottles depends on whether the bottles are plastic or glass. Plastic bottles for sampling or for laboratory use may be polyolefins (including conventional polyethylene, linear polyethylene, polypropylene, polyallomer, and "TPX" polymethylpentene), polycarbonate, or Teflon. Among this group, polypropylene, polycarbonate, "TPX" polymethylpentene, polyallomer, and Teflon FEP may be autoclaved repeatedly at 121°C for 15 minutes. To allow pressure equalization and prevent the plastic bottles from collapsing during autoclave sterilization, the screw caps should not be tightly closed.

Glass sample bottles with plastic screw caps must also be autoclaved at 121°C for 15 minutes since plastic materials used in the screw cap and liner may not withstand the high temperatures of dry heat sterilization. Glass sample bottles with ground-glass covers should be sterilized by dry heat (2 hours at 170°C); this method ensures complete drying of the dechlorinating agent solution added to each bottle before sterilization. The thin film of dechlorinating agent thus formed cannot be accidentally spilled when the open sample bottle is manipulated during sample collection.

Individual Glassware Items

Many glassware items (flasks, beakers, graduated cylinders, etc.) commonly used in the bacteriological laboratory require sterilization before use. Since contact with steam during autoclaving may introduce chemical contamination from boiler water to carefully washed glassware items, dry heat sterilization (1-hour exposure to 170°C) is recommended. Metal foil or paper covers of durable material (kraft paper or equivalent parchment type) must be secured over open ends of these items to maintain sterility when the items are removed from the sterilizer and during storage before use. Precautions for dry heat sterilization include the following: (a) glassware must be completely dry; (b) the oven must be cool when these items are inserted, and (c) the oven should be allowed to cool to near room temperature before removing glassware because sudden or uneven cooling may cause glassware to fracture.

Glassware and Inoculating Equipment in Metal Containers

Glass pipets, Petri dishes, and single service inoculating equipment are generally stored in stainless steel or aluminum containers suitable for dry heat sterilization. To ensure adequate heat penetration for sterilization of these glassware items and single-service transfer loops or applicator sticks, 2-hour exposure to dry heat at 170°C is required. Metal containers of stainless steel resist heat damage and last longer than those constructed of aluminum although either is acceptable.

Disposable, single-service hardwood applicators should only be dry heat sterilized because steam sterilization may generate wood distillate products that may be toxic to bacteria.

Dilution Water Blanks

Dilution water blanks are sterilized by autoclaving at 121°C for 15 minutes. Trays of these blanks should be packed loosely to permit even exposure to flowing steam and to ensure that those blanks in the center of the load reach sterilization temperature. Screw caps or rubber stopper closures should be slightly loosened to permit pressure equalization during autoclaving. Some loss of dilution water volume may result from either evaporation or boil-over when steam pressure is rapidly reduced during the autoclave exhaust cycle. Adjusting the timing of the steam exhaust cycle should correct this problem, but consistent volume losses of the dilution blank that are greater than 2 percent will require dispensing 101- or 102-ml volumes of dilution water to compensate for the approximate 1- to 2-ml water loss during autoclaving. Adjusting the volumes of the dilution blanks before sterilization will eliminate the need for the laboratory staff to adjust the final volume to 99 ml by pipetting sterile dilution water into each of the deficient water blanks after autoclaving.

Plastic Culture Dish Resue

Shortages of plastic culture (Petri) dishes used in the MF tests, which are generally considered to be a disposable item, may, at times, make it necessary to consider their reuse when no substitute glass Petri dishes are available. The procedure for reuse consists of discarding old cultures and hand cleaning the top and bottom sections in a mild household dish detergent. Following a rinse and air-drying, the dish sections are ready for sterilization. Since this plastic material cannot withstand heat exposure during autoclaving, other sterilization methods must be used. Plastic culture dishes may be sterilized by soaking individual top and bottom sections in 70 percent ethanol for 30 minutes, then placing these parts on a clean towel to drain and air dry before reassembly. A more convenient approach is to expose the interior portion of these dishes to UV light for 5 minutes and reassemble for storage or immediate reuse.

Plastic culture dishes may also be subjected to gas sterilization in a Cryotherm chamber with 12 percent ethylene oxide at 120°C for a 4-hour contact period. As a safety precaution relative to the explosive nature of pure ethylene oxide in air at certain concentrations, a mixture of ethylene oxide and carbon dioxide of decreased explosive and flammable properties must be used. Controls must be established to ensure the adequacy of the flushing procedure to remove traces of ethylene oxide from the culture dishes. Such a test for sterility would be:

1. Prepare a known coliform suspension of approximately 100 to 150 organisms per 1 ml in buffered dilution water for test use within 20 minutes. Prepare five replicate pour plates immediately, using 1-ml aliquots of the test suspension.
2. Place 1-ml aliquots of the suspension in each of five plastic culture dishes sterilized by the ethylene oxide procedure and hold for 10 minutes.
3. Place 1-ml aliquots of the suspension in each of five glass culture dishes sterilized by dry heat and hold for 10 minutes.
4. After 10 minutes add plate count agar, swirl to mix, and allow to solidify.
5. Incubate 48 hours at 35°C, then determine densities.
6. Counts between cell suspensions held in the two sets of culture dishes should be within 15 percent of the initial dilution and within 10 percent of each other.

When these sterilization procedures are used, it will be necessary to select one plate from each batch sterilized for use as a sterilization control. Standard plate count agar is added to the dish, mixed by gentle rotation, solidified, then incubated at 35°C for 48 hours. No bacterial growth should appear on the control plate if sterilization was accomplished with the procedure chosen.

Flame Sterilization

Wire inoculating loops and needles are sterilized by heating in an ordinary Bunsen burner flame until the wire glows red hot. When sterilizing wire loops, take care to avoid creating a hazardous aerosol that can result from splattering of residual culture broth. Gradually draw the inoculating wire through the burner flame, and thus allow the broth to evaporate to dryness before the loop is actually in the flame. Immediately before using loops and needles, allow them to cool to near room temperature to avoid heat killing the bacterial cells during transfer of growth from broth, agar, or the MF surfaces.

Forceps and spatulas are generally surface sterilized by dipping in alcohol and then burning off the residual alcohol to incinerate any attached bacteria. Direct heating of forceps and spatulas until red hot destroys the temper of the metal and may brand the MF during manipulation.

Laboratory Water Quality

Laboratory water should be free of toxic or nutritive substances that could influence survival or growth of bacteria and viruses. This special water supply should, in addition, be free of microorganisms that might contribute inhibitory substances to dilution water and media, pyrogens that are deleterious in animal injections, and virus tissue cultures and substances that interfere with sensitive chemical measurements. Many factors may influence the quality of a laboratory distilled water supply: (a) design of the distillation apparatus; (b) source of raw water; (c) condition of the deionizing column, if used; (d) state of the carbon filter; (e) storage chamber for reserve supply; (f) temperature of stored supply; and (g) duration of storage before use. These factors may contribute contaminating substances to the distilled water including metal ions from the distribution system; ammonium hydroxide, hydrochloric acid, and other fumes from the laboratory; chlorine from the tap water supply; and carbon dioxide from the air. Therefore, distilled water pH may vary.

The processes used to produce distilled water evolved through the years on an empirical basis. As a result, little attention has been directed toward proper engineering of a system to yield a product that is completely satisfactory for biological applications. This problem is common to both public health laboratory distilled water systems and to a large number of water stills used by commercial suppliers of bottled distilled water.

The best distilled water system utilizes stainless-steel construction, but adequate systems may also be built from quartz, vicor, or Pyrex glass, in that order of preference. A tin-lined system is the least desirable because it requires periodic maintenance to replace hardware sections that have lost tin plating and, thereby, expose copper or other base metal to contact with the distilled water. All connecting plumbing should be stainless steel, Pyrex, or special plastic pipes made of polytetrafluoroethylene (PTFE) material (5). Polyvinyl chloride (PVC) is a major contaminant in high-quality laboratory water systems and should not be used for connecting plumbing (6). Storage tanks should be stainless steel, fiberglass, or suitable plastic (PTFE), and protected from dust contamination.

The ideal conventional distilled water system should be regulated to divert the first 10 percent of the initial daily output to waste, the middle portion to a storage tank for high-quality distilled water requirements, and the remaining portion to another storage tank for general distilled water uses. Careful control of the distillation process should be maintained to minimize splash-over and flash-over of undistilled water or volatile contaminants.

The input source water should be passed through a deionizing column and a carbon filter before distillation. With careful maintenance of these two columns, much of the inorganic and organic constituents of tap water will be removed. However, not maintaining these columns can actually result in a lower quality input water than that from the original tap water source. Charcoal filters have been found to support the growth of bacteria to alarmingly high counts. These charcoal beds concentrate both bacteria and organic nutrients present from source water at low concentrations (7). Therefore, a quality check should be made once a month to monitor the general bacterial population level for excessive growth. Such growth necessitates appropriate treatment or reactivation of the carbon column to control any buildup of heat stable, antibiotic substances. Use of a disposable in-line MF cartridge would alleviate this problem and ensure a higher quality input water.

Recharge or regeneration of deionizers must receive prompt attention whenever the water quality shows either a loss of chemical suitability or a sudden decrease in bacterial quality as measured by a standard plate count (35°C incubation for 48 hours on standard plate count agar). The general bacterial population in a city water supply used as source water contributes a variety of organisms to the resin beds. In such an environment, a bacterial population can quickly develop to densities ranging from 6,000 to 1,000,000 organisms per ml (8) because of the accumulation of organic and inorganic material, adequate moisture, and large surface area for attachment.

Limiting bacterial discharge from the resin bed is a partial benefit derived from a commercial system that uses disposable cartridges for prefiltering the source water, followed by organic adsorption, deionization, and finally membrane filtration. Municipal tap water can be processed at a rate of 20 gallons per hour, and the deionized water produced will be in the 10 megohm conductivity range, with no particulate matter larger than 0.45 micron. Although this type of membrane filtration unit will limit the passage of bacteria and particles larger than 0.45-micron size, smaller microbial forms and waste products (dissolved metabolites) produced by the bacterial populations in the resin beds pass through the filter into the product water.

Distilled water can be contaminated by nutrients derived from many sources. Some common causes include organic flash-over during distillation, continued use of exhausted carbon filter beds, deionizing columns in need of recharging, dust contamination and chemical fumes entering the stored supply, and the storage of distilled water in glass bottles not thoroughly cleaned before use. Laboratory supplies of high quality distilled or deionized water should be protected from strong sunlight to prevent algal growth capable of producing organic nutrients or antibacterial metabolites.

Excellent quality water can also be produced by a reverse osmosis system used in conjunction with a series-connected deionizing column. The quality improves as the number of deionizer columns incorporated in the system is increased. Water having a resistivity of 4.5 megohm-cm can be routinely produced when one deionizer is used; adding a second unit in series will increase the resistivity to 10 megohm-cm, or better. (The lower the concentration of ionic contaminants in the water, the higher its resistivity.) To maintain this high quality water, the first deionizer should be recharged once each month and the second unit, once each year. The favorable rate of recharge is possible only because the reverse osmosis process removes approximately 90 percent of the ions. The reverse osmosis membrane may require changing only once each year depending on the quality of the input water. Water produced by this system should be stored in either fiber glass or stainless steel tanks.

CHEMICAL QUALITY CONTROL FOR DISTILLED AND DEIONIZED WATERS

Various physical and chemical parameters must be monitored at scheduled intervals to ensure the continued production of high-quality distilled or deionized water. One essential laboratory water quality measurement is conductivity, particularly when conductivity measurements are made at various points in the system train. Resistivity measurements reflect the presence of ionized material (inorganic metals, salts, and bases) but do not distinguish between the presence of toxic or nontoxic metallic ions. This measurement also does not reveal any organic contaminants that may be present. The specific resistivity of freshly prepared, good quality water should exceed 0.5 megohm-cm at 25°C (equivalent to electrical conductivity of 1.0 ppm as NaCl) or 2 microhms-cm.

Since most source waters used for the production of distilled water are city water supplies, the distillate may show increased concentration of ammonia and chloramines (9). Removal of free chlorine by distillation of municipal water may be difficult because it apparently forms an azeotrope with water at pH values greater than 5.5. As a result, flash-over of chloramines is frequently a serious problem in water plant laboratories where freshly chlorinated water is used for distillation. When this problem is encountered, suitable dechlorination procedures for the laboratory water supply must be instituted. In other instances, trace concentrations of volatile, short-chain fatty acids have been found in distilled water (10). Additional chemical tests and use of an AutoAnalyzer will yield supplemental information for routine quality monitoring on the chemical impurities of a high quality laboratory water supply. Although such procedures may detect various undesirable impurities, they provide no measure of the relative biological toxicity of the impurities.

BIOLOGICAL SUITABILITY TEST FOR DISTILLED AND DEIONIZED WATER

Biological toxicity or nutritive releases from distilled and deionized water supplies can be measured by a suitability test (11) that compares the growth response of *Enterobacter aerogenes* in a minimal growth medium prepared with the test water and the growth response of the organism in a high quality water control. This test, described in *Standard Methods* (12), is useful in evaluating the quality of distilled water from newly installed or repaired water distillation systems and as a periodic check on the condition of existing stills, storage tanks, and laboratory water distribution systems. The microbiological quality of laboratory water should be evaluated at a frequency that will ensure the continued production of a high-quality product. Experience indicates that the suitability test should be performed annually, with additional tests following line alterations, equipment repairs, or cleaning of the distribution network to individual laboratory areas. (For a description of medium, see Chapter 6.)

Some state laboratory systems find it impractical to require small laboratories to perform this quality control test and, therefore, offer this service through the central state laboratory. This approach has produced greater data reliability in monitoring the microbiological quality of all laboratory water supplies within the state network of certified laboratories. When conducting the distilled water suitability test, several samples can be evaluated at the same time with little additional work since a large portion of the time for this test involves reagent and culture preparation. When this service is offered, the sampling schedule should be timed so that a series of test samples are examined on a given day to obtain maximum laboratory efficiency. Test samples over 48 hours old should not be examined to avoid possible leaching of impurities from plastic sample containers or from the cap liner. Used plastic bottles are not recommended for shipment of laboratory water samples because of low-level chemical residuals from previous bottle uses that might affect test results. Clean borosilicate glass bottles are the preferred sample container.

When toxic or nutritive organic complexes are present in distilled water, the first indication may be erratic replicate results for pour plate or membrane filter counts and irregular growth in certain minimal nutrient culture media. Erratic plate counts may also result from improper washing procedures that leave toxic detergent residues on glassware items. Erratic MF counts for a replicate series may also be due to poor quality MF's or absorbent pads used in the experiment. If the washing procedure and detergent are proven satisfactory or the MF products are of acceptable quality, then the distilled water supply becomes a prime suspect and should be investigated.

Enterobacter aerogenes is the test organism because it can grow in minimal nutrients and does not require complex amino acids or other additives necessary for *Escherichia coli* or *Streptococcus faecalis*. *Pseudomonas aeruginosa* and other pseudomonads can also grow in the presence of minimal nutrients and could be insensitive to inhibitory factors produced by resident pseudomonads already present in the unknown distilled water.

The minimal medium requirements to support a moderate growth of *Enterobacter aerogenes* include: carbon source (citrate), nitrogen source (ammonium sulfate), salt mixture (magnesium, calcium, iron, and sodium), and a buffer (phosphate) solution to maintain a favorable medium pH. All chemicals used in preparation of nutrient stock solutions should be analytical reagent (AR) grade. This is particularly important in preparing potassium dihydrogen phosphate (KH_2PO_4) since some brands have significant amounts of chemical impurities.

Only borosilicate glassware may be used in this test and all items must receive a final rinse in freshly redistilled water from a glass still before dry heat sterilization. This is necessary because the sensitivity of the test depends upon the cleanliness of all items used (sample containers, flasks, tubes, and pipets).

All stock solutions must be boiled 1 to 2 minutes to kill vegetative bacterial cells (12). The stock solutions can then be stored in sterilized glass-stoppered bottles at refrigerator temperature (5°C) for a maximum period of 1 month. The inorganic salt stock solution will develop a slight turbidity within 3 to 5 days as ferrous salt oxidizes to the ferric salt. Stock solutions that develop heavy chemical turbidity or bacterial contamination should be discarded and a new stock solution prepared. The buffer solution may also develop turbidity because of bacterial contamination; when this occurs, it should likewise be discarded. The distilled water test sample should be filtered through a 0.22-micron porosity MF or, alternatively, boiled for 1 minute to kill vegetative bacterial cells. Longer boiling time should be avoided to prevent changing the chemical composition of impurities in the unknown sample.

An appropriate aliquot of an *Enterobacter aerogenes* suspension is added to each flask so that the final cell concentration will be 30 to 80 bacterial cells per ml. Bacterial densities below 30 cells per ml produce ratios that are not consistent, whereas densities greater than 100 cells per ml result in decreased sensitivity to impurities in the test water. An initial bacterial count is made by plating 1 ml of each culture flask in standard plate count agar to verify the cell density range and to check for gross contamination of the sample or media. The culture flasks and pour plates are incubated at 35°C for 24 ± 2 hours. After incubation, the initial plate counts are recorded and final plate counts are prepared from each flask; dilutions of 1, 0.1, 0.01, 0.001, and 0.0001 ml are used. After incubating the final set of pour plates for 24 ± 2 hours, these cultures are examined and those plates having 30 to 300 colonies are counted.

After the bacterial density for each flask is determined from a selection of plate counts within the 30 to 300 colony range, a series of ratios are calculated to evaluate the growth results. The ratio for growth-inhibiting substances is:

$$\frac{\text{Colony count per ml, Flask B}}{\text{Colony count per ml, Flask A}}$$

Ratio values between 0.8 and 1.2 indicate that toxic substances are not present; whereas values less than 0.8 are positive indication of bacterial toxicity. Since the test is also sensitive to the presence of nutritive contaminants, ratios up to 3.0 are permissible because no significant unstabilizing growth effects are created in buffered dilution water.

When the suitability test results indicate that contamination of the laboratory water has reached a toxic level, the distillation system must be disassembled, cleaned, and carefully inspected. Hardware sections where tin plating has been lost can result in distilled water being exposed to copper or other base metal used in the manufacture of the equipment.

All connecting plumbing should be stainless steel, Pyrex, or special plastic pipes made of PTFE material. Use of dissimilar metal connections can cause electrolysis and corrosion, which can result in metal ion toxicity. Inspection of stills may indicate that there is a need for more frequent still "clean-up" to minimize chemical residue buildup.

Before scheduling a shut-down for still maintenance and clean up, provisions must be made for a reserve supply of distilled water to supplement storage tank capacity. This may necessitate purchasing an additional storage tank to be incorporated into the system for emergency use during repair and routine maintenance of laboratory water systems. More uniform quality of laboratory water is ensured if a central supply of distilled or deionized water is used rather than water from several stills in different laboratories. The key to successful maintenance of high quality laboratory water is having a staff microbiologist or chemist assigned the task of routine surveillance of the system for quality control and adequate production. This responsibility should include: daily checks of conductivity; chemical analysis for selected chemical impurities that are related to source water quality; periodic recharge of the demineralizer; establishment of production capacity that will ensure a stand-by reserve supply; yearly inspection of valves, electrical heating elements, storage tank, and distribution lines for defects; and finally, a yearly distilled water suitability test to confirm the suitability of the laboratory water supply.

DILUTION WATER

Bacteriological examination of polluted waters necessitates the usage of serial dilutions of the water samples to obtain a bacterial density range within the statistical limits of any quantitative procedure. In the multiple tube concept, the sample must be proportionally diluted so that a series of positive and negative culture reactions is obtained. Colony counts on MF's or in agar pour plates must also be limited in density because of restricted surface area; this also necessitates appropriate dilution of high-density samples to achieve a suitable bacterial population that may be more accurately counted. In addition, the MF and agar pour plate procedures demand particulate-free diluent so that discrete colony growth and visibility are not impaired. Particulate matter in the agar pour plate procedure may contribute significantly to counting error.

The ideal diluent is one that causes no change in the bacterial density and does not depress the recovery of attenuated organisms. Many diluents have been proposed—some recommended for selected organisms and others specifically recommended for use with water, food, or medical specimens. The major reasons for divergent opinions on the proper choice of diluent generally relate to the physiological state of the microorganisms that must be recovered from a given sample. Thus, evaluation of a suitable diluent for water samples must be related to the condition of bacteria in natural water samples, not to the response of pure cultures of bacteria or to results obtained on food products or medical specimens.

The chemical content of water varies from trace concentrations of nutritive or toxic substances in some groundwater or in high mountain streams, to the high nutrient concentrations found in food processing wastes and in industrial effluents. Therefore, microbial survival in a selected diluent can be quite variable and is influenced by suspension time, temperature, pH, osmotic gradient, buffering, chelating capacity, and trace concentrations of magnesium, calcium, and iron ions in the diluent formulation.

Distilled water is not recommended for water sample dilution because it is deficient in essential trace metal ions and in buffering and chelating capacities (13,14). Tap water modified in various ways (charcoal filtered or containing 0.1 percent sodium thiosulfate) has been used but the results have drawn divergent interpretation because the inorganic salt content and the effect of water treatment varies widely from one public water supply to another (15,16). Sterile sea water used as a diluent is also suspect because of the chance occurrence of heat stable antagonistic agents. Physiological saline (0.85 percent sodium chloride in distilled water) may preserve the viability of some species of bacteria (including organisms damaged by phenol) but has been reported to be bactericidal to *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Streptococcus pyogenes* (16,17). Minimal nutrient water, generally prepared as a 0.1 or 0.05 percent peptone solution in distilled water, has also been used as a diluent; it is particularly useful in the recovery of attenuated organisms from food products (18). Results from the investigation of peptone water as a diluent for water samples showed that at room temperature bacterial multiplication could occur when the time between sample dilution and plating exceeded 40 minutes (Table 1). For this reason, using 0.1 percent peptone as a diluent requires that the 30-minute limit on processing serial dilutions be closely followed. The 0.1 percent peptone water diluent may be superior to phosphate dilution water in the recovery of attenuated organisms from industrial wastes or from stream samples that have high concentrations of heavy metal ions. The pH of peptone water diluent should be adjusted to pH 6.8.

If bacterial growth with minimal lag is to be achieved in the bacteriological examination of high quality natural waters, some degree of mineralization, corresponding to that of natural water, is necessary. Phosphate-buffered dilution water comes close to fulfilling this requirement, as shown by the data in Table 2. Sterilized source waters were used as diluents and compared with phosphate-buffered dilution water used in this study. These data and those presented in Table 1 illustrate the need for prompt sample processing through serial dilutions within 30 minutes so as to reduce significant changes in the bacterial density at room temperatures. When longer contact times are necessary for special research studies, loss of viable cells in diluent suspensions can be suppressed for periods up to approximately 2 hours by packing the inoculated dilution blank in ice.

Stock potassium phosphate buffer solution (34.0 grams KH_2PO_4 per liter distilled water) should be adjusted to pH 7.2. After the addition of 1.25 ml stock buffer and 5.0 ml magnesium sulfate solution (50 grams $MgSO_4 \cdot 7\,H_2O$ per liter distilled water) to 1 liter of distilled water, the final pH after autoclaving should be 7.2 ± 0.1. The addition of magnesium sulfate to phosphate buffer dilution water improves the recovery of organisms with metabolic injury induced by high-quality water or by waters containing significant concentrations of heavy metal ions (14). Since

TABLE 1. SURVIVAL OF BACTERIA IN VARIOUS DILUENTS STORED AT ROOM TEMPERATURE

Sample	Type of diluent	Initial no. of bacteria*	Percent change in no. of bacteria				
			20 min	40 min	1 hr	2 hr	3 hr
Ohio River (Water intake)	0.05% Peptone	110	+11	−23	− 2	+ 11	+ 36
	0.1% Peptone	100	0	+26	+ 7	+ 48	+ 83
	Phosphate buffered	100	−22	+11	+35	− 13	+ 67
Ohio River (Public Landing)	0.05% Peptone	110	+ 2	0	−14	+ 18	+170
	0.1% Peptone	90	+19	+18	+16	+106	+214
	Phosphate buffered	70	−10	−11	−44	− 39	+ 4
Pure cultures, coliform IMViC types:							
+ + − −	0.05% Peptone	120	+ 3	− 8	+ 3	+ 19	+ 79
	0.1% Peptone	140	−20	−13	−13	+ 3	+ 48
	Phosphate buffered	110	+13	+25	+16	+ 2	+ 11
− − + +	0.05% Peptone	110	+ 4	− 6	+16	+ 5	+ 86
	0.1% Peptone	110	+ 5	+22	+16	+ 24	+218
	Phosphate buffered	130	−15	−39	− 1	− 20	− 33
− + − +	0.05% Peptone	90	− 7	−11	−11	+ 21	+210
	0.1% Peptone	90	−17	−20	−16	+ 4	+141
	Phosphate buffered	70	+16	+ 6	+23	+ 49	+ 36
Streptococcus faecalis	0.05% Peptone	55	− 4	+11	+ 7	− 7	+ 13
	0.1% Peptone	60	−18	−20	− 8	+ 2	+ 8
	Phosphate buffered	53	+ 8	−11	+ 2	0	+ 4
Streptococcus durans	0.05% Peptone	55	−22	−15	+ 2	0	+ 6
	0.1% Peptone	63	−30	−22	−22	− 10	+ 6
	Phosphate buffered	55	−20	−26	− 2	− 51	− 93
Staphylococcus aureus	0.05% Peptone	56	−11	+13	+ 2	+ 11	+ 7
	0.1% Peptone	58	− 4	− 4	− 2	+ 16	+ 19
	Phosphate buffered	52	+23	−23	+12	− 62	− 90

*Standard plate counts per 1 ml (35°C incubation for 24 hours).

dilution water is generally recognized to be a harsh environment for survival of attenuated bacteria found in chlorinated waters and sewage effluents, the addition of magnesium sulfate should alleviate this problem. When buffered dilution water is prepared in dilution bottles or culture tubes of poor quality glass, the pH after sterilization may become more alkaline (pH 7.5 or higher) because of substances leaching out of the glass. Such glass containers must be removed from service and replaced with a high-quality glassware (borosilicate formulation or equivalent) since increased alkalinity of dilution water has a bactericidal effect on cell suspensions.

TABLE 2. BACTERIAL POPULATION SURVIVAL IN AUTOCLAVED SOURCE WATER AND BUFFERED DILUTION WATER*

Sample source	Autoclaved source water			Buffered dilution water		
	Plate count, per ml†	Percent change		Plate count, per ml†	Percent change	
		15 min	30 min		15 min	30 min
White Clay Creek, S.D.	208	− 4	+ 8	220	−11	−14
Lake Michigan, Ill.	124	+35	+37	137	−14	−16
Goose Creek, S.C.	152	− 3	− 2	146	− 3	− 4
Kansas River, Kan.	151	−13	−19	140	− 7	−15
Saugus River, Mass.	388	− 1	+ 5	382	+ 7	+ 1
Sangamon River, Ill.	213	+ 5	+ 6	201	+14	+ 9
Ohio River, Ohio	27	− 7	− 4	30	− 3	−13

*Selected data from Butterfield (15).

†Plate counts incubated 37°C for 24 hours.

When turbidity due to microbial contamination is observed in the stock buffer, fresh stock buffer solution should be prepared. Such turbidity may be caused by many different kinds of organisms (bacteria, yeast, fungi). These organisms are capable of survival and growth in the presence of the minimal concentrations of nutrients present in buffered dilution water. Microbiological analysis of contaminated stock buffer solution generally shows large numbers of *Pseudomonas* and *Achromobacter* species. Once species of *Pseudomonas* have become established in dilution water, their antagonistic action toward other organisms may adversely affect test results (19).

Place 25- to 30-ml portions of freshly prepared, sterilized (by MF filtration) stock buffer solution into previously sterilized screw-cap test tubes; or place the same amount of buffer solution in screw-cap test tubes and autoclave the solution and tubes for 15 minutes at 121°C. Store the sterilized tubes and solution at 5° to 10°C. Sterile stock buffer is then available in small volumes as needed and if chance contamination should occur during the removal of stock buffer, only a small volume of stock buffer solution from a single tube needs to be discarded. A similar approach can be used to store the stock magnesium sulfate solution used in conjunction with stock buffer and distilled water to make buffered dilution water.

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GUIDELINES ON LABORATORY MATERIALS PREPARATION

Cleaning Glassware

Dishwasher Manufacturer ________________ Model ________
Thoroughly washed in detergent at 160°F, cycle time ________________
Rinsed in clean water at 180°F, cycle time ________________
Final rinse in distilled water, cycle time ________________
Detergent brand ________________
Washing procedure leaves no toxic residue .... ________
Glassware free from acidity or alkalinity .... ________
Glassware clean, free of film deposits .... ________

Sterilization Procedures

Tube media and reagents sterilized 121°C for 12 to 15 minutes .... ________
Tubes packed loosely in baskets for uniform heating and cooling .... ________
Timing began when autoclave reached 121°C .... ________
Total exposure of carbohydrate media to heat not over 45 minutes .... ________
Media removed and cooled as soon as possible after sterilization .... ________
MF media parboiled for 5 minutes .... ________
Reagents and media additions sterilized by MF filtration .... ________
MF presterilized or 121°C for 10 minutes, then exhaust .... ________
MF filtration equipment sterilized at: 121°C for 15 minutes; UV for 2 minutes, or in boiling water .... ________
Individual glassware items sterilized 1 hour at 170°C (dry heat) .... ________
Pipets, Petri dishes, inoculating loops in boxes, sterilized 170°C for 2 hours ________
Dilution water blanks sterilized 121°C for 15 minutes .... ________
Wire loops, needles, forceps, and spatulas flame sterilized .... ________

Quality of Laboratory Water

System analysis:
  Still manufacturer ________________ Construction material ________
  Demineralizer ________________ Recharge frequency ________________
  Protected storage tank ________________ Construction material ________________
  Supply adequate for all laboratory needs .... ________
Chemical quality control:
  Resistivity exceeded 0.5 megohms-cm at 25°C ________ pH ________
  Free from traces of heavy metals and chlorine .... ________
  Free from organics .... ________
Biological suitability:
  Free from bactericidal compounds as measured by bacteriological suitability test ________________ Test ratio ________________
  Bacteriological quality of water measured once each year by suitability test; sooner, if necessary .... ________
Systems maintenance:
  Inspected, repaired, cleaned out .... ________
  Reservoir stand-by supply provided .... ________
  Adequate surveillance program .... ________

Dilution Water

pH of stock phosphate buffer solution 7.2 .... ________
Fresh stock buffer prepared when turbidity appeared .... ________
Stock buffer autoclaved and stored at 5° to 10°C .... ________
1.25 ml stock potassium phosphate buffer solution and 5.0 ml magnesium sulfate solution added per 1 liter distilled water .... ________
Dispensed to give 99 ± 2 ml or 9 ± 0.2 ml after autoclaving .... ________
pH of sterile phosphate buffered water 7.2 ± 0.1 .... ________

CHAPTER VI
CULTURE MEDIA SPECIFICATIONS

The preparation of culture media for laboratory use has undergone considerable advancement from the early, laborious art of processing crude animal and plant materials into peptones, suppressing agents, and agar substrates plus the further refining of textile dyestuffs into usable indicator agents. Various commercial suppliers now manufacture a wide variety of the basic ingredients for culture media formulations. However, because of convenience and labor-saving advantages, most laboratories use commercially prepared dehydrated media for large-volume, routine, bacteriological procedures. The need for small quantities of biochemical test media on an infrequent basis or the limited staff and media preparation facilities of small laboratories may justify the use of sterile tubes or culture plates of prepared media available from commercial outlets even though the cost per test is higher. Ampuled media or preweighed vials of dehydrated media may be used for convenience in a laboratory performing only few tests and also in conjunction with portable MF kits because of convenience and compact storage and because less preparation is needed in the field.

MEDIA PREPARATION

Dehydrated culture media are available as finely ground powders, granules, or tablets (1-3). The choice is largely dictated by cost, availability, and convenience; however, finely ground powders are most frequently used. These preparations dissolve quickly, but because they are hygroscopic, long-term storage must be avoided in humid environments. Media processed into granules may have a better shelf life because they are relatively less hygroscopic. Using prepared media tablets permits the easy preparation of small fixed volume batches of media, but the tabulation process must not use any binding substances not specified in the basic formula.

Regardless of the commercial processing method, these products are best reconstituted by slowly adding the appropriate weighed quantities to approximately half of the total volume of distilled water. Freshly distilled or boiled distilled water, or equivalent, should be used because old supplies of distilled water absorb sufficient gases to alter the final medium pH. Only chemically clean glassware or stainless steel utensils should be used to prepare and dispense media into culture tubes or bottles. The mixture of distilled water and medium should be gently agitated by hand or by magnetic stirrers to ensure rapid dissolution. Dissolution is also aided by preheating the distilled water to approximately 45° to 50°C. After thorough mixing, the container is rotated and the remaining volume of distilled water is added slowly to wash residual powder from the inner

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walls of the container. Most reconstituted culture broths will go into complete solution with careful mixing. Some media preparations, however, may have a normal turbidity resulting from insoluble materials in indicator dyes, low solubility of selective agents (tetrathionate), or creation of colloidal particles in agar preparations.

When a medium formulation includes agar, gelatin, or cystine, the water-medium mixture should be allowed to soak for about 5 minutes to obtain a more uniform suspension. Follow this by applying heat to bring about complete solution and to permit the medium to be dispensed in culture tubes or bottles. Finally, sterilize. Agar may be dissolved in several ways; the easiest is to place the flask containing the ingredients in a boiling water bath to dissolve the agar medium into a uniform solution. Large quantities of agar medium may be more effectively dissolved by 15 to 20 minute's exposure to flowing steam in an autoclave, set to operate without pressure buildup, or in a steamer. Gelatin media are best dissolved by heating in a boiling water bath. Agar and gelatin media must be in complete solution before being dispensed into culture tubes or bottles and then sterilized. If the agar is not in complete solution before being dispensed into individual tubes or bottles, it will not be distributed uniformly and, in some cases, the agar concentration may be so low that the medium will fail to solidify after cooling to room temperature.

DISPENSING CULTURE MEDIA

Once a medium is dissolved, it should be dispensed into appropriate culture tubes or bottles and promptly sterilized by appropriate procedures (4). To avoid bacterial growth in this material, which can alter medium pH and introduce toxic metabolic byproducts, the total time from media preparation to sterilization should not exceed 2 hours. Refrigerating unsterilized prepared media overnight before sterilization is undesirable because bacterial activity will not be completely suppressed.

Broth or melted agar medium is generally dispensed into culture tubes or bottles by means of an automatic pipetting machine set to deliver the appropriate volume. Such equipment must be thoroughly rinsed immediately after use to avoid carry-over of dyes, carbohydrates, and selective inhibitory chemicals to subsequent medium dispensed by this system. When an agar medium is dispensed, its temperature should be maintained above 60°C so that the agar remains fluid long enough to be passed through the system and be adequately flushed out before it solidifies in the pipetter.

Culture tubes and bottles should be covered with metal caps, plastic plugs, or screw-cap closures, as required, immediately after dispensing the medium. Screw-cap closures should be loosely fitted until after autoclaving so that the pressure within the culture tube or bottle equilibrates to the autoclave pressure during sterilization. Large bottles, in particular, may crack when removed from the autoclave if the caps are completely tightened.

MEDIA pH MEASUREMENTS

The electronic pH meter available for use in media preparations must be calibrated in the range of intended use by means of a precision buffer standard. Do not assume that the pH meter scales are linear throughout their total pH range. As an illustration, a pH meter calibrated with pH 9.0 buffer may read low by 0.4 pH unit when used to measure a solution at or near pH 7.0. Since most bacteriological media used in the water laboratory are near pH 7.0, the standard buffer chosen to calibrate the pH meter at daily intervals should be pH 7.0. Colorimetric methods or pH paper strips impregnated with indicator dyes are not acceptable because color changes are masked by dyes in the media such as BGLB broth, eosin methylene blue agar, M-Endo broth, M-FC broth, and other selective media formulations.

When media pH deviates from the established tolerance of ± 0.1 pH units, immediately check the pH meter calibration for drift. If the meter is functional, check for preparation and sterilization errors. If the problem is not due to the factors above, then poor quality distilled water or a poor quality commercial medium should be suspected.

Adjustment of the medium pH before sterilization requires the use of small volumes of either a base (1 N sodium hydroxide) to shift the pH higher or an acid (1 N hydrochloric acid) to shift the pH lower. Allowance must also be made for a shift in pH (usually 0.1 to 0.2 pH unit lower) during autoclaving, so that the final pH value will meet the recommended values.

MEDIA STORAGE

Supplies of commercial dehydrated media do not remain stable indefinitely. Certain constituents will decompose and create byproducts that adversely affect the sensitivity and selectivity of differential media. If heavier components sift to the lower depths of finely divided powder batches, it may result in a nonhomogeneous mixture. Imperfect bottle seals may allow moisture to be taken up by dehydrated media powders that are very hygroscopic. In laboratories that are not equipped with air conditioning, bottles of dehydrated media should be stored upside down. Containers stored this way have a self-sealing effect around the screw-cap liner that will retard media decomposition. Once sufficient moisture gains entry, the powder becomes caked into a hard mass or, in some cases, develops a viscous consistency. In either case, such products undergo changes that can alter their usefulness in culturing bacterial strains and may alter the biochemical responses expected.

As new supplies are received, each container should be dated and older packages used first. Laboratory personnel should inventory stock supplies every 3 months. At the time of inspection, those supplies affected by moisture contamination should be discarded. Media supplies used most frequently should be purchased in quantities estimated to last no longer than 1 year, preferably purchased on a 6-month basis. Those media that are used infrequently or in very small quantities daily or those that are very hygroscopic should be purchased in quarter-pound sizes rather than in 1- or 5-pound (454 or 2270 gram) quantities. Despite the lower cost per unit when purchased in bulk quantities, open or unsealed packages of media with slow turn-over may deteriorate before substantial amounts are used. Discarding partially used bulk packages represents a greater economic loss than would have occurred if small size packages of the specific medium were bought.

Prepared culture media should be stored in an area that affords protection from direct sunlight, contamination, and excessive evaporation. Prepared media may be stored in cold rooms, if sealed in plastic bags or other sealed containers, or at ambient temperature. Frost-free refrigerators may cause excessive media evaporation on storage beyond 1 week. Cold storage areas must not contain volatile solvents whose absorption into media may be toxic to bacteria. After storage at low temperatures, prepared tubes of fermentation media must first be held overnight at room or incubator temperature to check for microbial contamination before being inoculated. Equilibration of media to room temperature after cold storage will also reduce erroneous results due to absorbed atmospheric gases. These gases would otherwise be released during test incubation and could mask the detection of gas produced by bacterial fermentation (5). All media showing turbidity or gas bubbles after warming up from cold storage temperature should be discarded.

Culture media stored at ambient laboratory temperature must be protected from strong light. If media containing light sensitive dye substances (BGLB broth, M-Endo broth, M-FC medium, etc.) are not protected from direct sunlight, or fluorescent light decomposition of the dye substances in these media will result in a significant reduction in their suppressive action on noncoliform organisms.

Extended storage of sterile media will increase the risk of contamination, fading of indicator color intensity, precipitation or excessive evaporation all of which can drastically alter performance of these preparations. Agar slants and pour plate preparations of selective media may begin to lose moisture in storage. This can result in the creation of dry or rough surfaces that are undesirable for optimum microbial growth. Media evaporation and contamination develop more quickly using loose-fitting caps, cotton plugs, and Petri dish containers. Therefore, unless screw cap culture tubes or tight fitting culture dishes are used, limit media production to quantities calculated to be used within a 1-week period.

MEDIA QUALITY CONTROL

In general, using commercially prepared dehydrated media is preferable to preparing media for routine use from basic ingredients; commercial products are less subject to the minor variations in chemical composition that may be introduced when weighing individual components. This simplified, single weighing of a preformulated medium should produce greater uniformity in composition and also reduce preparation time.

Although commercial dehydrated media are generally acknowledged to be more desirable than laboratory preparations, the manufacturer may substitute ingredients such as peptones of different composition from those originally used or include a bile complex or other material that is not of equivalent selectivity to the one recommended by the medium developer (6-7). This problem can be further complicated where the medium formulation must include some biological dyes (basic fuchsin, brilliant green, analine blue, rosalic acid, etc.) that are technical grade products having varying percentages of active dye and "inert" material.

Media manufacturers attempt, through their quality control programs, to evaluate the differences in ingredients and select from among their own products those components that give the best equivalence to the original formulation. As new lots of each medium are produced, they should be submitted to an adequate quality control testing program by the manufacturer to ensure optimum recovery and colony differentiation. Failure to meet specifications for sensitivity, selectivity, and colony differentiation (where applicable) should be sufficient reason to prevent a specific batch of medium from being released through commercial outlets for laboratory use. However, media quality control may represent a substantial part of operational costs. Attempts to reduce this essential function result in inadequate or ineffective quality control and may result in an increased risk that batches of substandard media will be released for laboratory use.

Although a quality check is made of these commercial products (8,9), it appears to be inadequate, at times. Poor quality total coliform sheen development and significant reductions in coliform recovery on M-Endo medium have been observed by several laboratories in recent years. Apparently, the use of poor grades of basic fuchsin and inadequate dye-sulfite balance in the medium are responsible. Basic fuchsin may differ in dye content, both from lot to lot and from manufacturer to manufacturer; this makes it essential to standardize the fuchsin-sulfite proportion used each time a new lot of dye is employed. Variations in intensity of the blue color of fecal coliform colonies on M-FC medium may be caused by residual acidity in absorbent pads or MF's and also from unsatisfactory lots of aniline blue used in the commercial preparation of this medium. The intensity and structure of bile salt crystals that precipitate on fecal coliform colonies relate to the type of bile salts complex incorporated in the medium. Formulations of commercial media containing sodium azide (M-Enterococcus, KF, and PSE agars) have an approximate shelf life of 2 years after production, because of the deleterious effects created by the slow decomposition of the azide compound. For these reasons, it is desirable for the laboratory to establish a quality control analysis on each new lot of medium purchased—to compare it with a lot of the same medium known to be satisfactory in terms of differential qualities and sensitivity.

MEDIA EVALUATION

The analysis of a medium for selectivity and adequate quantitative recovery must be based on appropriate water samples, which can be altered by dilution or by dosing with selected organisms. The use of pure cultures suspended in buffered dilution water does have some value in determining recovery rates for those particular strains, but provides no information on the interaction effects of a mixed bacterial flora and of the water chemistry on test medium performance. Choose an appropriate well water or lake sample or dose a potable water sample so that the colifrom density ranges from 5 to 10 organisms per 100 ml and the standard plate count (35°C for 48 hours) ranges from 1,000 to 10,000 organisms per ml. Many poorly treated, marginal, public, and private potable water supplies meet these sample specifications. Bottled waters dosed with 5 to 10 coliforms per 100 ml may be another suitable sample source since a standard plate count of these waters frequently demonstrates a high general bacterial population but few or less than one coliform per 100 ml.

1. Prepare a batch of test medium appropriate to the testing procedure (fermentation tube media for the MPN or a broth or equivalent agar for the MF procedure).

2a. When evaluating a multiple tube test medium, examine the selected water sample (containing 5 to 10 coliforms per 100 ml) by inoculating 25 tubes of the double-strength test medium lot (lactose broth or lauryl tryptose broth) with 10 ml sample aliquots and a second set of tubes prepared from a known satisfactory lot of the same type of medium. Incubate at 35°C for 24 to 48 hours, and confirm all positive tubes from each set using brilliant green lactose bile broth (incubated at 35°C for 24 to 48 hours) or EC broth (incubated at 44.5°C for 24 hours). If the medium being tested is brilliant green lactose bile or EC broth, prepare two sets of presumptive positive tubes and confirm using the unknown and known lots of the same confirmatory medium. Record all positive confirmed test results for each set. Satisfactory results for the test medium lot should be within ± 1 positive tube of the control medium lot.

2b. When evaluating a MF test medium, examine the selected water sample (containing 5 to 10 total coliforms or fecal coliforms per 100 ml) by incubating one set of 10 replicate 100-ml sample filtrations on the test medium (M-Endo or M-FC) and a second set of 10 replicate 100-ml sample portions on a known satisfactory lot of the same medium. Incubate at the appropriate temperature for the test and count total coliform or fecal coliform colonies on all membranes in each set. Verify all coliform colonies by transferring individual colonies to lactose or lauryl tryptose broth for gas production at 35°C, then confirm in BGLB broth at 35°C or EC broth at 44.5°C. Total all verified coliform counts for each set of 10 replicates. For a satisfactory test medium, the total verified coliform or fecal coliform colonies on the 10 membrane replicates should be within ± 5 colonies of the total colonies verified from the known medium lot (10). Poor verification obtained on the test medium lot when compared with the reference medium may indicate that traces of other fermentable carbohydrates have contaminated the formulation during manufacture or that overheating the medium during preparation caused lactose hydrolysis.

2c. When evaluating standard pour plate agar (SPC agar), prepare one set of 20 replicate pour plates using the unknown medium lot and another set of 20 replicate pour plates using a known satisfactory lot of SPC agar. Select a water sample containing a bacterial population, either undiluted or diluted, of 100 to 150 organisms per ml. Avoid pipetting 0.1 ml sample portions to minimize pipetting errors (11). Incubate all pour plates at 35°C for 48 hours if the sample is from municipal drinking water supplies or 35°C for 72 hours if bottled water samples are used.

Following incubation, determine colony counts for each set of pour plates, then calculate the geometric mean value for each set of 20 replicate counts. Colony counts on a satisfactory test medium lot should be within ± 10 percent of the counts obtained on a known reference medium lot.

MEDIA pH RECORDS

The pH of all batches of culture media should be checked after sterilization and the pH of each batch recorded with the date and medium lot (control) number. As an absolute minimal requirement, the pH of at least one batch of sterilized medium from each new bottle of commercial medium must be determined to ensure its quality. By monitoring final medium pH, a check can be made on possible errors in weighing, excessive heating, and sterilization resulting in lactose hydrolysis, chemical contamination, or deterioration of ingredients that might occur during storage after stock packages are opened.

GENERAL CHEMICALS

All chemicals used in the preparation of culture media must be ACS (American Chemical Society) or AR grade. This is particularly important since some chemical impurities found in commercial and other lower grades of chemicals can be present in large enough concentrations to suppress or inhibit bacterial growth.

BACTERIOLOGICAL DYES

Dyes may differ in biological activity from lot to lot and from manufacturer to manufacturer. Information on dye technology indicates that differences between lots of a given dye are related to the dye content produced, the dye complex mixture present, and the amount of inert, insoluble residues remaining in the product. Therefore, it is important that all dyes used in the preparation of culture media be purchased from lots certified by the Biological Stain Commission for bacteriological use.

STANDARD CULTURAL MEDIA SPECIFICATIONS

Media described in this section have been recognized as essential to the measurement of total coliform and fecal coliform populations. The standard plate count is included because of its application to monitoring the general bacterial quality of drinking water in distribution systems and in bottled water supplies.

Lactose Broth (12)

Beef extract	3.0 gram
Peptone	5.0 gram
Lactose	5.0 gram
Distilled water	1,000 ml

Final pH after sterilizing (121°C for 12-15 min):
  Single strength pH 6.9 ± 0.1
  Double strength pH 6.7 ± 0.1
Single strength dehydrated medium, 13.0 grams per liter

The final concentration of ingredients in lactose broth after the addition of the water sample must equal normal-strength broth. This presents no problem when water-sample volumes of 1 ml or less are added to 10 ml of single-strength broth. When 10 ml volumes of a water sample are examined, however, the lactose broth must be double strength so that the dilution of broth by the 10 ml sample will result in single-strength broth. Thus, each milliliter in the planted tube contains the equivalent of 0.013 gram of dehydrated medium. A more dilute lactose-broth concentration may result in a significant reduction in the recovery of attenuated coliform organisms and a concurrent increase in slow fermentation reactions. The net result is a reduced sensitivity for coliform detection.

Do not dispense medium volumes that are less than 10 ml into the larger culture tubes (25- × 150-mm) used for examining 10-ml sample portions. Such a practice would make these broth preparations subject to air entrapment during handling or as the result of the rapid pipetting of 10-ml sample volumes into the broth tube. Air entrapment creates a false judgment that gas entrapment from bacterial fermentation has occurred.

Where irregular volumes are used, the quantity of dehyrated lactose medium needed (grams per liter) may be calculated as follows:

$$\frac{(X)\ (\text{ml broth})}{\text{T.V.}} = 13.0$$

where:

X = number of grams per liter in lactose broth
ml broth = milliliters of broth per sterile tube
T.V. = total volume of sterile broth plus water sample added per tube, or

$$X = \frac{(13)\ (\text{T.V.})}{\text{ml sterile lactose broth per tube}}$$

Therefore, lactose broth with 35 ml of broth and 100 ml of water sample should contain 50.1 grams dehydrated lactose medium per liter. Table 3 illustrates the number of grams per liter of dehydrated medium required to maintain a final single-strength concentration of lactose broth when used with 1-, 10-, or 100-ml sample test portions.

TABLE 3. CONCENTRATIONS OF DEHYDRATED LACTOSE BROTH REQUIRED TO MAINTAIN THE PROPER CONCENTRATION OF INGREDIENTS

Inoculum, ml	Amount medium in tube, ml	Vol. medium and inoculum, ml	Dehydrated-lactose broth required, gram/liter
1	10 or more	11 or more	13
10	30	40	17.3
10	20	30	19.5
100	50	150	39.0
100	35	135	50.1
100	20	120	78.0

Lauryl Tryptose Broth (13)

Tryptose	20 gram
Lactose	5 gram
Dipotassium hydrogen phosphate (K_2HPO_4)	2.75 gram
Potassium dihydrogen phosphate (KH_2PO_4)	2.75 gram
Sodium chloride	5 gram
Sodium lauryl sulfate	0.1 gram
Distilled water	1,000 ml

Final pH after sterilizing (121°C for 12-15 min)
Single strength pH 6.8 ± 0.1
Double strength pH 6.7 ± 0.1

Single strength dehydrated medium, 35.6 grams per liter

The final concentration of ingredients in lauryl tryptose broth, after the additon of the water sample, must equal normal strength broth. When 10-ml volumes of a water sample are examined, lauryl tryptose broth must be double strength so that the dilution of the broth by the 10-ml sample will result in single-strength broth. Thus, each milliliter in the planted tube contains the equivalent of 0.0356 gram per ml of dehydrated medium. More dilute lauryl tryptose broth concentrations may result in significant reduction in the recovery of attenuated coliform organisms and a significant increase in slow fermentation reactions. The net result is a reduced sensitivity for coliform detection.

Do not dispense medium volumes that are less than 10 ml into the larger culture tubes (25- × 150-mm) used for examining 10-ml sample portions. Such a practice would make these broth preparations subject to air entrapment during handling or as the result of the rapid pipetting of 10-ml sample volumes into the broth tube. Air entrapment creates a false judgment that gas entrapment from bacterial fermentation has occurred.

Where irregular volumes are used, the quantity of dehydrated lauryl tryptose broth needed (grams/liter) may be calculated as follows:

$$\frac{(X)\ (\text{ml broth})}{\text{T.V.}} = 35.6$$

where:

X = number of grams per liter in lauryl tryptose broth
ml broth = milliters of broth per sterile tube
T.V. = total volume of sterile broth plus water sample added per tube, or

$$X = \frac{(35.6)\ (\text{T.V.})}{\text{ml lauryl tryptose broth per tube}}$$

Table 4 illustrates the number of grams per liter of dehydrated lauryl tryptose broth required to maintain a single strength concentration of lauryl tryptose broth when used with 1-, 10-, or 100-ml sample test portions.

TABLE 4. CONCENTRATION OF DEHYDRATED LAURYL TRYPTOSE BROTH REQUIRED TO MAINTAIN THE PROPER CONCENTRATION OF INGREDIENTS

Inoculum, ml	Amount medium in tube, ml	Vol. medium and inoculum, ml	Dehydrated lauryl tryptose broth required, grams/liter
1	10	10	35.6
10	20	30	53.4
10	30	40	47.3
100	50	150	106.8
100	35	135	137.1

Brilliant Green Lactose Bile Broth (14)

Peptone	10 gram
Lactose	10 gram
Dehydrated Oxgall	20 gram
Brilliant green	0.0133 gram
Distilled water	1,000 ml

Final pH 7.2 ± 0.2 after sterilizing (121°C for 12-15 min)
Single strength dehydrated medium, 40 grams per liter

Dispense brilliant green lactose bile broth in no less than 10-ml volumes per tube to ensure complete filling of the fermentation vial and to partially submerge this vial at least halfway.

EC Medium(15)

Tryptose or Trypticase	20 gram
Lactose	5 gram
Bile Salts mixture or Bile Salts No. 3	1.5 gram
Dipotassium hydrogen phosphate (K_2HPO_4)	4 gram
Potassium dihydrogen phosphate (KH_2PO_4)	1.5 gram
Sodium chloride	5 gram
Distilled water	1,000 ml

Final pH 6.9 ± 0.1 after sterilizing (121°C for 12-15 min)
Single strength dehydrated medium, 37 grams per liter

Dispense EC medium in no less than 10-ml volumes per tube to ensure complete filling of the fermentation vial and to partially submerge this vial at least halfway.

Eosin Methylene Blue Agar(16)

Peptone	10 gram
Lactose	10 gram
Dipotassium hydrogen phosphate (K_2HPO_4)	2 gram
Agar	15 gram
Eosin Y	0.4 gram
Methylene blue	0.065 gram
Distilled water	1,000 ml

Final pH 7.1 ± 0.1 after sterilizing (121°C for 12-15 min)
Single strength dehydrated medium, 37.5 grams per liter

The eosin methylene blue (EMB) agar of Holt-Harris and Teague, used for isolation of intestinal pathogenic bacteria, should not be used in the total coliform MPN completed test procedure. This medium contains saccharose in addition to lactose; the saccharose can result in a significant increase in noncoliform organisms appearing as coliform colonies on this formulation of EMB agar. Levine EMB agar is recommended for confirmation or isolation of coliform bacteria from positive broth cultures as an essential screening and purification step in the MPN completed test.

Endo Agar (17)

Peptone	10 gram
Lactose	10 gram
Dipotassium hydrogen phosphate (K_2HPO_4)	3.5 gram
Agar	15 gram
Sodium sulfite	2.5 gram
Basic fuchsin	0.4 gram
Distilled water	1,000 ml

Final pH 7.4 ± 0.1 (no autoclaving)
Single strength dehydrated medium, 41.5 grams per liter

Sterilization of the complete Endo agar at 121°C for 15 minutes is not recommended. Excessive heat destroys the sodium sulfite; this destruction results in poor sheen development on coliform colonies. Therefore, dissolve the agar preparation in a boiling water bath, cool to 45°C, and pour the necessary plates. When this medium is properly prepared, all coliform colonies growing on the surface from streak inoculation will have a golden metallic sheen.

Another approach to the preparation of an excellent streak plate Endo agar requires adding 1.5 percent agar to M-Endo medium. The medium is then heated in a boiling water bath to dissolve the agar completely, and pour plates are prepared with the usual precautions against contamination and allowed to harden.

M-Endo Medium (18)

Tryptone or polypeptone	10 gram
Thiopeptone or thiotone	5 gram
Casitone or trypticase	5 gram
Yeast extract	1.5 gram
Lactose	12.5 gram
Sodium chloride	5 gram
Dipotassium hydrogen phosphate (K_2HPO_4)	4.375 gram
Potassium dihydrogen phosphate (KH_2PO_4)	1.375 gram
Sodium lauryl sulfate	0.05 gram
Sodium desoxycholate	0.1 gram
Sodium sulfite	2.1 gram
Basic fuchsin	1.05 gram
Distilled water containing 20 ml of ethanol	1,000 ml

Final pH 7.2 ± 0.1 (no autoclaving)
Single strength dehydrated medium, 48 grams per liter

The addition of pure grain ethanol to a final concentration of 2 percent (V/V) to form alcohol esters is essential for the development of coliform colonies with a maximum sheen and with less tendency toward confluent growth. These esters tend to suppress significant numbers of noncoliform organisms that could otherwise develop on the medium. Denatured ethanol commonly available in the laboratory must not be used since the denaturant commonly employed is either methanol or propanol, both of which are toxic to coliforms.

When state laws or laboratory directives severely restrict the availability of pure ethanol, a stock supply of ethanol for use in the MF procedure may be technically denatured by adding a few grains of M-Endo powder. The trace amount of basic fuchsin present in the small amount of dehydrated powder turns the ethanol pink, does not adversely affect the M-Endo medium formulation, and nullifies the illegal use of the product for human consumption.

Excessive heating of M-Endo medium destroys or reduces its specificity. Therefore, the medium is heated only to the boiling point (as described in the section on Sterilization Procedures). As a general practice, only enough M-Endo medium is prepared to meet anticipated daily needs. However, surplus medium may be saved for use within a 96-hour period provided the medium is stored in the dark at 2° to 10°C. Protected storage in the dark is essential since M-Endo medium is sensitive to strong artificial light or to direct sunlight.

One formulation of Endo medium known as LES Endo agar may be prepared by adding 1.5 percent agar to 75 percent of the recommended grams of M-Endo powder per 100 ml of distilled water. The mixture is then heated in a boiling water bath to completely dissolve the agar and poured in plates (60-mm Petri dishes) for use with a MF procedure.

M-FC Broth (19)

Tryptose or biosate	10 gram
Proteose peptone No. 3 or polypeptone	5 gram
Yeast extract	3 gram
Sodium chloride	5 gram
Lactose	12.5 gram
Bile salts No. 3 or bile salts mixture	1.5 gram
Aniline blue	0.1 gram
Distilled water containing 10 ml of 1% rosolic acid salt reagent	1,000 ml

Final pH 7.4 ± 0.1 (no autoclaving)
Single strength dehydrated medium, 37 grams per liter

After the medium ingredients are in solution, 1 ml of a 1 percent rosolic acid salt reagent is added and the medium is heated to the boiling point (as described in the section on Sterilization Procedures). As a general practice, only enough M-FC broth is prepared to meet daily needs. However, surplus medium may be saved for use within a 96-hour period provided the medium is stored in the dark at 2° to 10°C.

The 1 percent rosolic acid salt reagent is prepared by dissolving 1 gram of rosolic acid in 100 ml of 0.2 N sodium hydroxide (0.8 gram NaOH in 100 ml distilled water). Do not autoclave this solution. Rosolic acid salt reagent should be stored at 2° to 10°C in the dark and must be discarded after 2 weeks or sooner if the solution changes color from dark red to muddy brown or if, after the addition of the rosolic acid, the prepared medium is not the proper color. Background color on the MF will vary from a yellowish cream to faint blue, depending on the age of the reagent. When rosolic acid salt has been prepared within an hour or two of its addition to the medium, it does have a differential effect on some of the nonfecal coliform colonies. This phenomenon has been shown by the development of yellow and red nonfecal coliform colonies from samples of canal waters in the Chicago area. What the organisms were is not known, but the important point is that only blue colonies verified as fecal coliforms.

In M-FC broth, aniline blue is the indicator system used to detect lactose fermentation, and development of the blue colony color does not depend upon the addition of the rosolic acid salt reagent. The sodium salt of rosolic acid is added to the medium to supress a variety of nonfecal coliform organisms, which may grow at the elevated temperature and which are common to some specific source waters and the first flush of stormwater runoff. Without the inhibitory effect of the rosolic acid salt, a substantial background growth of white- to gray-colored colonies may develop and cause interference with the discrete growth of the blue-colored fecal coliform colonies.

M-VFC Holding Medium (20)

Vitamin-free casitone	0.2 gram
Sodium benzoate	4.0 gram
Sulfanilamide	0.5 gram
Ethanol (95%)	10.0 ml
Distilled water	1,000 ml

Final pH 6.7 ± 0.1 (no autoclaving)
Single strength dehydrated medium, 4.7 grams per liter

Warm to dissolve all ingredients, then sterilize the medium by filtration through an 0.22-micron MF. If only 100 ml of the medium are prepared, it is easier to add the vitamin-free casitone as 2 ml of a 1:100 aqueous solution. Store the finished medium at 2° to 10°C, and discard any unused portions after 1 month's storage.

M–7-Hour Agar (21)

Proteose peptone No. 3 or polypeptone	5.0 gram
Yeast extract	3.0 gram
Lactose	10.0 gram
Mannitol	5.0 gram
Sodium chloride	7.5 gram
Sodium lauryl sulfate	0.2 gram
Sodium desoxycholate	0.1 gram
Brom cresol purple	0.35 gram
Phenol red	0.3 gram
Agar	15.0 gram
Distilled water	1,000 ml

Adjust final pH to 7.3 ± 0.1; approximately 0.35 ml of 0.1 N NaOH is required (no autoclaving).
Single strength dehydrated medium, 41.45 grams per liter

Heat the medium in a boiling water bath to dissolve the agar. After solution is complete, heat for an additional 5 minutes and then place in a

44.5°C water bath to temper heat before pouring the plates. Dispense 4 to 5 ml of the agar into 50- × 12-mm tight-fitting culture dishes, and allow to solidify. The medium may be stored at 2° to 10°C for periods up to 30 days before use.

M-PA Agar Base (22)

L-lysine hydrochloride	5.0 gram
Sodium chloride	5.0 gram
Yeast extract	2.0 gram
Xylose	2.5 gram
Sucrose	1.25 gram
Lactose	1.25 gram
Phenol red	0.08 gram
Ferric ammonium citrate	0.8 gram
Sodium thiosulfate	6.8 gram
Agar	15.0 gram
Distilled water	1,000 ml

Autoclave at 121°C for 15 minutes; cool mixture to between 55° to 60°C; adjust pH to 7.2 ± 0.1; and add the following dry antibiotics:

Sulfapyridine	176.0 mg
Kanamycin	8.5 mg
Nalidixic acid	37.0 mg
Cycloheximide (Actidione)	150.0 mg
M-PA agar base	1,000 ml

Dispense medium in 3-ml quantities to 50- × 12-mm Petri plates. Poured plates of the medium can be stored at 2° to 10°C for 1 month.

KF Streptococcus Agar (23)

Proteose peptone No. 3 or polypeptone	10.0 gram
Yeast extract	10.0 gram
Sodium chloride	5.0 gram
Sodium glycerophosphate	10.0 gram
Maltose	20.0 gram
Lactose	1.0 gram
Sodium azide	0.4 gram
Brom cresol purple	0.015 gram
Agar	20.0 gram
Distilled water	1,000 ml

Final pH 7.2 ± 0.1 (no autoclaving)
Single strength dehydrated medium, 76.4 grams per liter

Heat the medium in a boiling water bath to dissolve the agar. After solution is complete, continue heating for an additional 5 minutes. Cool medium to between 50° and 60°C and add 1 ml of sterile aqueous 1 percent solution of 2, 3, 5-triphenyltetrazolium chloride (available from either Difco or BBL) per each 100 ml of medium. Adjust the pH of the final medium to 7.2 with 10 percent sodium carbonate, if necessary. The fluid medium may be stored up to 4 hours in a water bath at 45° to 48°C before preparing plates.

For use with the MF technique, dispense 4 to 5 ml of the agar into 50- × 12-mm tight-fitting culture dishes, and allow to solidify. KF Streptococcus agar may be used immediately or stored in a cool, dark place and used any time within 2 weeks provided no dehydration has occurred.

Pfizer Selective Enterococcus (PSE) Agar (24)

Peptone C	17.0 gram
Peptone D	3.0 gram
Yeast extract	5.0 gram
Bacteriological bile	10.0 gram
Sodium chloride	5.0 gram
Sodium citrate	1.0 gram
Esculin	1.0 gram
Ferric ammonium citrate	0.5 gram
Sodium azide	0.25 gram
Agar	15.0 gram
Distilled water	1,000 ml

Final pH 7.1 ± 0.1 after sterilizing (121°C for 12-15 min)

Heat the PSE agar suspension to boiling; stir frequently to dissolve the medium completely. After solution, sterilize medium at 121°C for 15 minutes. The medium may be held at 45° to 50°C for up to 4 hours before preparing pour plates.

Plate Count Agar (Tryptone Glucose Yeast Agar) (25)

Tryptone	5 gram
Yeast extract	2.5 gram
Glucose	1 gram
Agar	15 gram
Distilled water	1,000 ml

Final pH 7.0 ± 0.1 after sterilizing (121°C for 12-15 min)
Single strength dehydrated medium, 23.5 grams per liter

Melt sterile supplies of plate count agar in a boiling water bath, and hold at 45°C until needed in the pour plate procedure. Remelting the plate count agar a second time or holding liquid supplies of this sterile agar for periods longer than 3 hours is undesirable because chemical precipitates may form and interfere with discernment of colony development.

Minimal Growth Medium for Suitability Test (26)

Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	0.005 gram
Ammonium sulfate	0.010 gram
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.004 gram
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.003 gram
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	0.004 gram
Sodium chloride	0.042 gram
Potassium dihydrogen phosphate	0.340 gram
Distilled water or test water	100 ml

Final pH 7.0 ± 0.2 (pH may vary as a reflection of test water)

Prepare the medium with high-purity chemicals. Sterilize medium by boiling 1 to 2 minutes or by MF filtration (0.22-micron pore size). Steam generated in autoclaving will introduce varying trace chemical impurities to this minimal growth medium for *Enterobacter aerogenes*. The medium may be prepared as per *Standard Methods* for the distilled water suitability test or as a complete medium for use in testing plastic items that might be releasing toxic substances.

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GUIDELINES ON CULTURE MEDIA SPECIFICATIONS

Media Preparation

Chemically clean glassware or stainless-steel utensils used .... ________
Freshly distilled or boiled distilled water used in media preparation .... ________
Complete solution obtained before dispensing to culture tubes or bottles .. ________
Total time from media preparation to sterilization less than 2 hours .... ________

Media pH Measurements

Electronic pH meter calibrated against appropriate standard buffer .... ________
Standard buffer brand ________________________________ pH_____ ___
pH of each sterile medium batch checked .... ________
A pH record of each sterile batch, the date, and lot number maintained ... ________
Causes for deviations beyond ± 0.1 pH unit investigated and
corrective action taken .... ________

Media Storage

Dehydrated media bottle kept tightly closed and protected from dust and
excessive humidity in storage areas .... ________
Dehydrated media discarded if discolored or caked .... ________
Dehydrated supplies dated ________ Shelf life not to exceed 1 year ____________
Sterile batches not in tubes or bottles with screw-caps used in less than 1 week ________
All media protected from sunlight .... ________
Media stored at low temperatures is incubated overnight and tubes with
air bubbles discarded .... ________

Media Quality Control

Media for detecting total coliforms, fecal coliforms, and standard plate
count quality tested .... ________
Media performance measured by natural water samples
MPN — comparative results of positive tubes .... ________
MF — comparative coliform colony count .... ________
Standard plate count — comparative replicate pour plates .... ________
Laboratory chemicals of analytical reagent grade .... ________
Bacteriological dyes certified for bacteriological use .... ________

Lactose Broth

Manufacturer________________________________Lot No.______________
Single strength composition, 13 grams per liter distilled water .... ________
Single strength, pH 6.9 ± 0.1; double strength, pH 6.7 ± 0.1 .... ________
Not less than 10 ml medium per tube .... ________
Medium, after 10-ml sample is added, contained 0.013 gram per
ml dry ingredients .... ________

Lauryl Tryptose Broth

Manufacturer________________________________Lot No.______________
Single strength composition, 35.6 grams per liter distilled water .... ________
Single strength pH, 6.8 ± 0.1; double strength pH, 6.7 ± 0.1 .... ________
Not less than 10 ml medium per tube .... ________
Medium, after 10-ml sample is added, contained 0.0356 gram per
ml of dry ingredients .... ________

Brilliant Green Lactose Bile Broth

Manufacturer________________________________Lot No.______________
Medium composition 40 grams per liter distilled water .... ________
Final pH, 6.9 ± 0.2 .... ________
Not less than 10 ml medium per tube .... ________

EC Medium

Manufacturer________________________Lot No.____________
Medium composition, 37 grams per liter distilled water .... ________
Final pH, 6.9 ± 0.1 .... ________
Not less than 10 ml medium per tube.................................. ________

Eosin Methylene Blue Agar

Manufacturer________________________Lot No.____________
Medium contains no sucrose; Cat. No. ________....................... ________
Medium composition, 37.5 grams per liter distilled water .... ________
Final pH, 7.1 ± 0.1 .... ________
Not less than 10 ml medium per standard Petri dish...................... ________

Endo Agar

Manufacturer________________________Lot No.____________
Medium composition, 41.5 grams per liter distilled water .... ________
Medium sterilized 10 minutes at 121°C or melted in boiling water bath
without further heating .... ________
Agar prepared from M-Endo plus 1.5 percent agar .... ________
Final pH, 7.3 ± 0.1 .... ________
Not less than 10 ml medium per standard Petri dish...................... ________

M-Endo MF Medium

Manufacturer________________________Lot No.____________
Medium composition, 4.8 grams per liter distilled water .... ________
Reconstituted in distilled water containing 2 percent ethanol .... ________
Pure ethanol used (not denatured) .... ________
Heated to boiling point, promptly removed and cooled .... ________
Final pH, 7.2 ± 0.1 .... ________
Stored in dark at 2° to 10°C .... ________
Unused medium discarded after 96 hours .... ________

M-FC Broth

Manufacturer________________________Lot No.____________
Medium composition, 3.7 grams per liter distilled water .... ________
Reconstituted in 100 ml distilled water containing 1 ml of a 1 percent
rosolic acid reagent .... ________
Stock solution of rosolic acid discarded after 2 weeks or when red color
changed to muddy brown .... ________
Heated to boiling point, promptly removed, and cooled .... ________
Final pH, 7.4 ± 0.1 .... ________
Stored in dark at 2° to 10°C .... ________
Unused medium discarded after 96 hours .... ________

M-VFC Holding Medium

Manufacturer________________________Lot No.____________
Medium composition, 4.7 grams per liter distilled water .... ________
Reconstituted in distilled water containing 1 percent ethanol .... ________
Final pH, 6.7 ± 0.1 .... ________
Stored in dark at 2° to 10°C .... ________
Unused medium discarded after 30 days .... ________

M-7-Hour Agar

Manufacturer________________________Lot No.____________
Medium composition, 41.45 grams per liter distilled water .... ________
Final pH adjusted to 7.3 ± 0.1 .... ________

Heated in boiling water bath to dissolve agar .... ________
Four to five ml dispensed into tight fitting culture dishes (50- × 12-mm) .. ________
Stored in dark at 2° to 10°C .... ________
Unused medium discarded after 30 days .... ________

M-PA Agar

Manufacturer________________________________Lot No.________________
Medium base composition, 39.68 grams per liter distilled water .... ________
Sulfapyridine (176.0 mg); Kanamycin (8.5 mg); nalidixic acid (37.0 mg); and Actidione (150.0 mg) added per liter of M-PA agar base .... ________
Final pH, 7.2 ± 0.1 .... ________
Four to five ml dispensed into tight fitting culture dishes (50- × 12-mm) .. ________
Stored in dark at 2° to 10°C .... ________
Unused medium discarded after 30 days .... ________

KF Streptococcus Agar

Manufacturer________________________________Lot No.________________
Medium composition, 76.4 grams per liter distilled water .... ________
Heated in boiling water bath to dissolve agar .... ________
Cooled to 50° to 60°C and 2,3,5 triphenyltetrazolium chloride added ...... ________
Final pH, 7.2 ± 0.1 .... ________
Placed in holding bath at 50° to 60°C for no more than 4 hours before pouring plates .... ________
Four to five ml dispensed into tight fitting culture dishes (50- × 12-mm) for MF use .... ________
Stored in dark at 2° to 10°C .... ________
Unused medium discarded after 30 days .... ________

PSE Agar

Manufacturer________________________________Lot No.________________
Medium composition, 57.75 grams per liter distilled water .... ________
Heated to boiling to dissolve the medium completely .... ________
Sterilized at 121°C for 15 minutes .... ________
Final pH, 7.1 ± 0.1 .... ________
Remelted in boiling water bath and placed in holding bath at 50° to 60°C for no more than 4 hours before pouring plates .... ________

Plate Count Agar

Manufacturer________________________________Lot No.________________
Medium composition, 23.5 grams per liter .... ________
Final pH, 7.0 ± 0.1 .... ________
Free from precipitates .... ________
Sterile medium not remelted a second time after sterilization .... ________
Placed in holding bath at 50° to 60°C for no more than 4 hours before pouring plates .... ________

________________Broth

Manufacturer________________________________Lot No.________________
Correct composition and pH .... ________
Purpose____

________________Agar

Manufacturer________________________________Lot No.________________
Correct composition and pH .... ________
Purpose____

CHAPTER VII
MULTIPLE TUBE COLIFORM PROCEDURES

The multiple tube coliform test has been a standard method for determining coliform quantification since 1936 (1). In this procedure, replicate tubes of lactose broth or lauryl tryptose broth are inoculated with decimal dilutions of a water sample. The coliform density is then calculated from probability formulas that predict the most probable number of coliforms necessary to produce certain combinations of gas-positive and gas-negative tubes in replicate decimal dilutions. McCrady first introduced the Most Probable Number (MPN) concept for estimating bacteria in 1915 (2), and this principle was later refined by Hoskins through the development of MPN tables (3).

TOTAL COLIFORM MPN PROCEDURE

During the evolution of the multiple tube procedure for the determination of total coliform density, it became apparent that three distinct test stages must be considered: the presumptive test, confirmed test, and completed test. In the presumptive test, the metabolic activity of attenuated bacteria are stimulated to greater vigor and a gross selection for lactose-utilizing organisms occurs. After incubation at 35°C, culture from each gas-positive presumptive tube is transferred into a tube of medium for the confirmed test. The confirmed test reduces the possibility of false gas-positive results occurring because of the metabolic activity of spore formers or the synergistic production of gas by some bacterial strains that, individually, cannot produce gas from lactose fermentation. To verify that the confirmed test does selectively eliminate all false positive tube results, it will occasionally be necessary to isolate these gas-producing bacteria and identify them as coliforms by the completed test procedure. The demonstration that the gas-producing isolates are gram negative, non-spore-forming, rod-shaped bacteria capable of gas production in a secondary lactose broth tube is conclusive evidence of the presence of coliforms and substantiates the reliability of the confirmed test.

CHOICE OF MULTIPLE DILUTIONS

Analysis of potable water by the multiple tube test consists of inoculating five tubes of presumptive medium with either 10-ml or 100-ml sample portions. In most cases, the inoculation of single-strength presumptive medium with 1.0- and 0.1-ml volumes of a potable water sample is of little additional value since the expected coliform density should be less than 10 organisms per 100 ml and is most often less than 2 coliforms per 100 ml.

When the multiple tube test is used for surface water quality studies, a minimum of three decimal dilutions must be used to ensure that quantita-

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tive data are obtained. In the absence of previous bacteriological data on the sample, it is necessary to use a five-decimal-dilution multiple tube test, to provide reasonable certainty of obtaining a break point between gas-positive and gas-negative tubes. Test results in which tubes at all dilutions are positive indicate only that the coliform density was greater than the upper limit of the test dilutions used and, therefore, are of no value in subsequent statistical analysis.

Decisions concerning choice of dilutions to be used in the multiple tube test must relate to information supplied with the sample. Suggested starting dilutions for a variety of samples are given in Table 5. When the water samples are part of a pollution survey or monitoring program, repeated sampling from the same locations may indicate the need to adjust the multiple tube decimal dilution series to obtain a more even split of positive and negative tube results. Repeated sampling will also establish an expected coliform range for each sample. This range may be fairly constant or may fluctuate with stormwater runoff, sudden discharge of industrial wastes, or sewage treatment bypass. In situations where the coliform density fluctuates widely, five decimal dilutions are necessary to prevent overruns of all positive tube results and loss of meaningful data. Samples that have more limited fluctuation may be tested using a three-decimal dilution multiple tube test.

TABLE 5. SUGGESTED STARTING DILUTIONS FOR MULTIPLE TUBE TOTAL COLIFORM EXAMINATIONS OF VARIOUS NATURAL WATER QUALITIES

Sample source	Starting dilutions (ml) for a three-dilution multiple tube test				
	10	1	0.1	0.01	0.001
Wells	x*				
Lakes	x- - - - - -	x			
Bathing beaches	x- - - - - -	x			
Creeks		x- - - - - -	x		
Rivers		x- - - - - -	x		
Sewages					
Chlorinated		x- - - - - -	x		
Secondary treatment		x- - - - - -	x		
Primary treatment			x- - - - - -	x	
Raw, municipal				x- - - - - -	x

*x = starting dilution; x - - - x = alternate choices

PRELIMINARY TEST PREPARATIONS

Preliminary preparation for MPN tests in the laboratory should include placing the lactose broth or lauryl tryptose broth tubes in test tube racks and labeling at least one tube in each row with the laboratory sample number. When samples to be examined require multiple dilutions, one or more tubes in each dilution should be labeled so as to avoid errors when confirming positive tubes or recording results.

The tube code shown in Table 6 may be helpful in minimizing identification errors and reducing the amount of wax pencil marks to be removed in the glassware washing process. Other coding schemes are acceptable provided they establish positive identification of sample volumes and are fully comprehended by all members of the laboratory staff assigned to examine water samples.

TABLE 6. A TEST TUBE CODING SCHEME

Inoculated volumes (ml)	Individual culture tube codes
10	A, B, C, D, E
1	a, b, c, d, e
0.1	a̲, b̲, c̲, d̲, e̲
0.01	1a, 1b, 1c, 1d, 1e
0.001	2a, 2b, 2c, 2d, 2e
0.0001	3a, 3b, 3c, 3d, 3e

PRESUMPTIVE TEST PROCEDURE

All water samples must be shaken vigorously, preferably in an inverted position, immediately before removing sample aliquots to inoculate a series of presumptive tubes in the multiple tube test. Vigorous shaking ensures a homogeneous distribution of bacteria suspended in the water sample and is of particular concern in the examination of highly turbid waters. Particulate matter in water rapidly settles out; this pulls suspended bacteria into the bottom sediment and, thereby, creates an uneven distribution of the bacterial population.

Greater accuracy in pipetting sample aliquots is achieved when 10-ml pipets are used to deliver only 10-ml amounts, 2-ml or 1-ml pipets are used to measure 1-ml portions, and only 1-ml pipets (graduated in 0.1-ml increments) are used to deliver 0.1-ml sample volumes. Sample volumes of 0.01 ml or smaller are prepared by decimal dilutions of 1 ml of the original water sample as shown in Figure 1. In withdrawing sample portions, the tip of the pipet should never be submerged more than 1 inch below the surface of the sample. This procedure minimizes the accumulative drainage from the exterior of the pipet into the medium and also prevents particles from being picked up from the bottom sediment that could introduce a significant clump of bacteria—a clump not representative of the bacteria in the water sample or of their distribution.

When adding sample aliquots into culture tubes, the pipet tip should be close to the surface of the broth to avoid impingement of droplet portions on the culture tube side walls and to ensure complete transfer of the sample aliquot into the culture medium. As a further precaution, it is suggested that each tube be mixed by a gentle shake or swirl as it is inoculated, so that the inoculum is mixed quickly and completely with the broth. After all the dilutions for one sample have been inoculated, the culture tube rack of inoculated presumptive broth tubes must be placed in the incubator within 30 minutes.

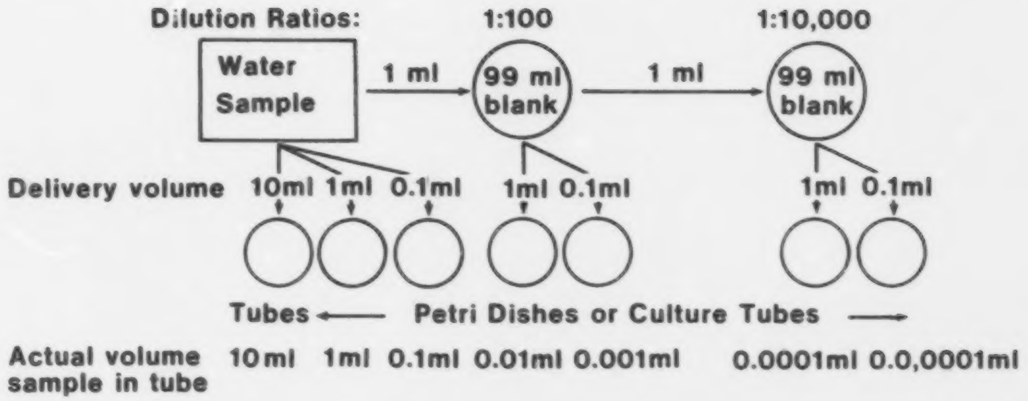
Dilution Ratios:
1:100
1:10,000
Water Sample
1 ml
99 ml blank
1 ml
99 ml blank
Delivery volume
10ml
1ml
0.1ml
1ml
0.1ml
1ml
0.1ml
Tubes
Petri Dishes or Culture Tubes
Actual volume sample in tube
10ml
1ml
0.1ml
0.01ml
0.001ml
0.0001ml
0.0,0001ml

Figure 1. Preparation Of Dilutions

The initial reading of presumptive tubes should be made after 24 hours ± 2 hours. Although this incubation time requirement may place the initial test observation in the afternoon, it is advantageous to gently shake all culture racks while in the incubator during the morning period. This procedure speeds up the release of gas into the fermentation tubes from the surrounding gas-saturated broth cultures. All tubes should be again shaken gently just before observations are recorded in the presumptive 24-hour column of the sample sheet or card.

Each tube should be examined carefully. Those tubes showing gas in the fermentation vial are recorded as positive (+), promptly submitted to the confirmatory procedure, and then discarded. Gas in any quantity (including tiny bubbles) is recorded as positive. It is essential that all positive tubes be confirmed at the end of the initial 24-hour period regardless of the amount of gas produced. The practice of not confirming positive lactose or lauryl tryptose broth tubes until the end of the 48-hour period is not acceptable. Because of the mixed bacterial flora competing with coliforms, particularly stressed coliforms, in the presumptive medium, tubes that are gas positive at 24 hours and contain coliforms frequently give negative results when confirmed after 48 hours incubation. Failure of 48-hour tubes to confirm may be due to low pH or to the antagonistic action of other organisms in the heterogeneous bacterial flora.

Lactose broth and lauryl tryptose broth yield equivalent recoveries of coliforms in the presumptive test. However, lauryl tryptose broth suppresses the development of aerobic sporeforming organisms that often ferment lactose with gas production. Therefore, when an analysis of laboratory data indicates that approximately 20 percent or more of the presumptive positive lactose tubes fail to confirm as coliforms, the use of lauryl tryptose broth should be investigated as a substitute presumptive medium. Parallel tests using lactose broth and lauryl tryptose broth on the variety of waters normally tested in the laboratory may reveal a marked reduction in false-positive presumptive tubes with the use of lauryl tryptose broth (4). This results in a savings of labor, materials, and time, plus a more rapid reporting of negative results. Evaluation of data from some types of water samples may, however, reveal little or no benefit in using lauryl tryptose broth to reduce false-positive presumptive tube occurrences. Therefore, since lauryl tryptose broth is somewhat more expensive, lactose broth may be preferred, all other factors being equal. The final choice of presumptive medium should await actual evaluation of a variety of water samples normally examined by the laboratory.

The amount of gas produced in presumptive tubes should not be the criterion for a positive test. Large-volume gas production in lactose or lauryl tryptose broth may be a result of several factors including the occurrence of noncoliform, spore-forming organisms. Conversely, active lactose-fermenting coliforms may be suppressed by the presence of specific soil organisms with the result that only a small bubble of gas may be produced within 48 hours. Antagonistic action of pseudomonads and other organisms (5-10) present in the bacterial flora can also suppress coliform growth so that the minimum concentration of cells (40 to 390 millions of cells per ml) required to produce visible gas in the presumptive medium is not obtained within the normal incubation time (11). The concentration of nitrate in some groundwater supplies may equal or exceed the 30 to 60 ppm range—a concentration shown to suppress gas production by coliform bacteria (12). For these reasons, it may be risky to disregard slow or weak lactose fermenters when assessing water quality.

Culture tubes not showing gas are recorded as a negative (-) results and returned for an additional 24-hour incubation period. At the conclusion of the second incubation period (24 hours), these cultures are again inspected for evidence of gas production. Any additional positive tubes are recorded and then submitted to the confirmatory procedure before discard. Those cultures showing no gas production are recorded as negative in the 48-hour presumptive column and then discarded.

CONFIRMED TEST PROCEDURE

Although the occurrence of gas production in the presumptive test indicates the probable presence of coliform bacteria, other organisms may be responsible for this gas production. Thus, all positive presumptive tubes must be submitted to a more selective test following enrichment in lactose or lauryl tryptose broth. BGLB broth used in the selective or confirmatory test can not be inoculated with the water sample directly because of significantly greater toxicity to attenuated coliforms.

The BGLB confirmatory procedure consists of transferring a small inoculum of culture from each positive presumptive tube to individual BGLB broth tubes and incubating them at 35°C for 48 hours. Gas production in BGLB broth tubes verifies that coliform bacteria are indeed present in the water sample examined.

When examining potable water, all gas-positive presumptive tubes are submitted to the confirmatory procedure. In water pollution and effluent examinations, however, the confirmation procedure may be modified if, after 24 hours of incubation, all five replicate tubes are gas positive for two or more consecutive sample volumes. With polluted waters or waste effluents, the set of five replicates representing the smallest volume of sample in which all tubes are gas positive is confirmed plus all other gas positive tubes from smaller sample volumes (higher sample dilutions) in which some tubes were positive and some were negative. This modification in the confirmatory procedure is predicated on the assumption that all five positive tubes in the lowest sample dilution would confirm if they were submitted to the confirmed test. Before transferring cultures from positive presumptive tubes to BGLB broth, the rack of cultures or each individual culture should be gently agitated to obtain a uniform bacterial suspension. Employ a sterile technique and, using an inoculating loop or an applicator, transfer an inoculum of gas-positive broth from the presumptive tube to a tube of BGLB broth labeled to correspond with the appropriate positive presumptive tube. Place each inoculated BGLB culture tube into the test rack position originally occupied by the presumptive positive tube. After making the transfers, the rack will probably contain some 24-hour negative presumptive tubes and the inoculated BGLB tube additions. Incubate all tubes at 35°C ± 0.5°C and check after 24 hours for gas production in the BGLB tubes and 48-hour presumptive tubes. Record BGLB tubes with gas production as positive and those tubes without gas as negative in the "24-hour confirmatory column" on a bacteriological report form. Record 48-hour presumptive tubes as negative or positive, and transfer growth from positive tubes into BGLB broth. Reincubate negative BGLB tubes for an additional 24 hours along with the newly inoculated BGLB tubes. Record results of the 48-hour BGLB tubes and any 24-hour BGLB tubes. Negative 24-hour BGLB tubes must be incubated an additional 24 hours and the results recorded before the test is concluded. Then calculate the MPN value from the combination of confirmed positive results and those negative confirmed and presumptive tubes. Record the calculated coliform density based on 100 ml of sample. When potable waters are examined, it is also permissible to report only the positive tube results rather than an MPN value. The entire test time may require a maximum of 96 hours when gas production is slow or a minimum of 48 hours if all tubes are negative in the presumptive test.

COMPLETED TEST PROCEDURE

The completed test is the reference standard for the multiple tube procedure. Since the confirmed test may yield positive reactions in the absence of the coliform group (false-positive test), it is essential that periodic comparisons be made with the reference standard to verify data reliability. The number of comparative procedures required to establish the validity of the confirmed test will be determined by the frequency of interferences from the water flora. Approximately 20 tests during each 3-month period should be sufficient where good agreement with the completed test is determined. For comparative testing, the samples selecfed should include all public water samples that are found to contain coliforms by the confirmed test. Since few municipal water samples will be found that contain measurable densities of coliforms, to obtain the minimum of 20 positive confirmed tests for processing through the completed test, use positive confirmed tests from raw water intakes for water treatment plants and private wells. The number of comparative tests should be increased whenever the sanitary interpretation of the results is questionable, and an investigation should be made to discover and correct the discrepancy. A quality control test of the BGLB may reveal poor medium selectivity. Additionally, the wrong concentration of BGLB medium or its exposure to light during storage or excessive heat during sterilization may be the cause of false-positive reactions in the confirmed procedure.

The completed test is applied to all gas-positive BGLB tubes in the individual test. It is permissible to assume that positive EC tube results from the fecal coliform portion of a double confirmation (BGLB tube for total coliform verification and EC tube for fecal coliform determination) are evidence of coliform presence. Therefore, the confirmatory tube should be recorded as a positive completed test response. All other confirmation positive BGLB tubes, which are not paralled with positive EC cultures, must be submitted to pure culture isolations on EMB or Endo agar streak plates (incubated at 35°C for 24 hours) then verified as lactose fermenting gram negative bacilli—the prerequisite to identification of coliforms in the completed test. In this procedure, an inoculum from each individual gas-positive confirmed tube is streaked to a plate of EMB or Endo agar (labeled to correspond with the confirmed tube) to obtain discrete colonies separated by approximately 0.5 cm or more. Since the observation of isolated colonies is mandatory for this procedure to be valid, subdivision of the plate area to permit confirmation of several positive BGLB tubes significantly restricts the probability of obtaining isolated colonies. Therefore, a maximum of two positive confirmatory tubes may be streaked onto one agar plate that has been divided into equal portions. After streaking, the agar plates are incubated at 35° ± 0.5°C for 24 ± 2 hours.

Following incubation, each EMB or Endo agar plate is examined for bacterial growth and colony appearance. Well-isolated colonies having a dark center (nucleated or "fisheye") are regarded as typical coliform colonies. These typical colonies may or may not have a metallic surface sheen. Colonies that are pink or opaque and not nucleated are considered atypical colonies but may be coliforms. Clear, watery colonies are not considered coliforms and are recorded as negative in the completed test.

To proceed with the completed test, an isolated colony (either typical or atypical) from each plate is then inoculated into tubes of lactose or lauryl tryptose broth to demonstrate lactose fermentation within 48 hours at 35°C and to agar slants to use in preparing a Gram stain after 18 to 24 hours incubation at 35°C.

The Gram stain must be prepared from an actively growing culture, preferably about 18 hours old and never more than 24 hours old. Preparations made from older cultures often result in unsatisfactory, irregular staining reactions. Clean glass slides, free of any trace of oily film, should be used. Use a wax pencil to divide the slide into squares no smaller than ½ inch. A drawing of the divided slide on the sample work sheet, with each square labeled with culture identification numbers, is useful for later reference when recording Gram stain results. Place one drop of distilled water on each divided portion of the slide, and use an inoculation needle to suspend a tiny amount of growth from a nutrient agar slant in each droplet. Mix the thin (almost invisible) suspension of cells with the tip of the inoculation needle, and allow the liquid to evaporate. Heat fix the smear by gently warming the slide over a flame. Do not overheat. This procedure prevents the bacterial cells from being washed off the slide during the staining procedure.

Stain the bacteria by flooding the area of the smear for 1 minute with crystal violet solution. Flush off the excess solution in gently running tap water and blot the slide dry with absorbent paper. Flood the smear with Lugol's iodine for 1 minute, and again rinse gently in running water and blot dry. Decolorize the smear by inclining it at a shallow angle and dripping 95 percent ethyl alcohol on it until no more crystal violet is removed—for approximately 15 to 30 seconds. Blot the smear dry and counterstain for 10 seconds with safranin solution. Wash in running water and blot dry. Place a drop of immersion oil on each of the stained squares of the slide preparation, and examine under the microscope using the oil immersion lens. The bacterial smear should contain nonspore-forming, rod (bacilli) shaped, red-stained cells (Gram negative), occurring singly, in pairs, or rarely in short chains. If this bacterial morphology is observed on the slide and the corresponding culture ferments lactose with gas production within 48 hours at 35°C incubation, coliforms are present and the completed test is recorded as positive. In those instances when the Gram-negative bacilli do not ferment lactose with gas production, or when the Gram stain shows that spore-forming cells are present, or if Gram-positive organisms or other morphological types of bacteria predominate, then that portion of the test is reported as negative. The positive-negative tube combinations from the entire test procedure (presumptive, confirmed, completed) can now be determined.

FECAL COLIFORM PROCEDURE

With only a little added effort, the fecal coliform test can be done by a multiple tube procedure (13, 14, Figure 2). In preparation for the test, tubes of EC broth are labeled to correspond with each gas positive tube of lauryl tryptose broth or lactose broth. Growth from each presumptive test gas-positive tube is transferred to a correspondingly labeled tube of EC broth with the use of a transfer loop or an applicator stick. Incubate the EC broth tubes at 44.5°C (± 0.2°C) for 24 hours in a waterbath with a gabled cover to reduce water and heat loss (see Elevated Temperature Incubation Requirements in Chapter VI). For optimum temperature regulation, the waterbath must have sufficient water depth to ensure complete immersion of the culture medium in all tubes. Air incubation cannot be substituted for water bath incubation because of the intolerable wide fluctuations in air temperature and slower temperature stabilization in tubes of culture medium introduced at the start of the incubation period. Inoculated tubes should be placed in the waterbath within 30 minutes following inoculation so that selective growth is related to elevated temperature exposure.

Following the 24-hour incubation period, the test tube racks of EC cultures are removed from the waterbath, shaken gently, and observed for gas production. Gas in any quantity is a positive test. Cultures with growth but no gas or tubes in which there is no visible indication of growth are recorded as negative. Calculate the most probable number based on the positive and negative tube combinations and report in terms of fecal coliforms per 100 ml.

Any direct inoculation of sample aliquots into EC tubes without preliminary enrichment in either lauryl tryptose or lactose broth is unsatisfactory. Research data obtained from parallel testing of 88 fecal samples showed that the average density of fecal coliforms detected was 24 percent when the enrichment procedure was not followed. Using the recommended enrichment before EC tube inoculations, however, produced a 90 percent recovery in the same specimens.

The need for the presumptive enrichment was also demonstrated in studies on the minimum *Escherichia coli* cell density necessary for gas production in EC broth. Most of 25 *E. coli* strains tested required from 1 to 20 viable cells to produce a gas positive reaction in EC broth when incubated for 24 hours at 44.5°C; however, three *E. coli* strains that required 500 or more viable cells per inoculum demonstrated that significant variability in the required number of cells may occur. An optimum cell density, generally in excess of 1,000 viable organisms, is ensured by

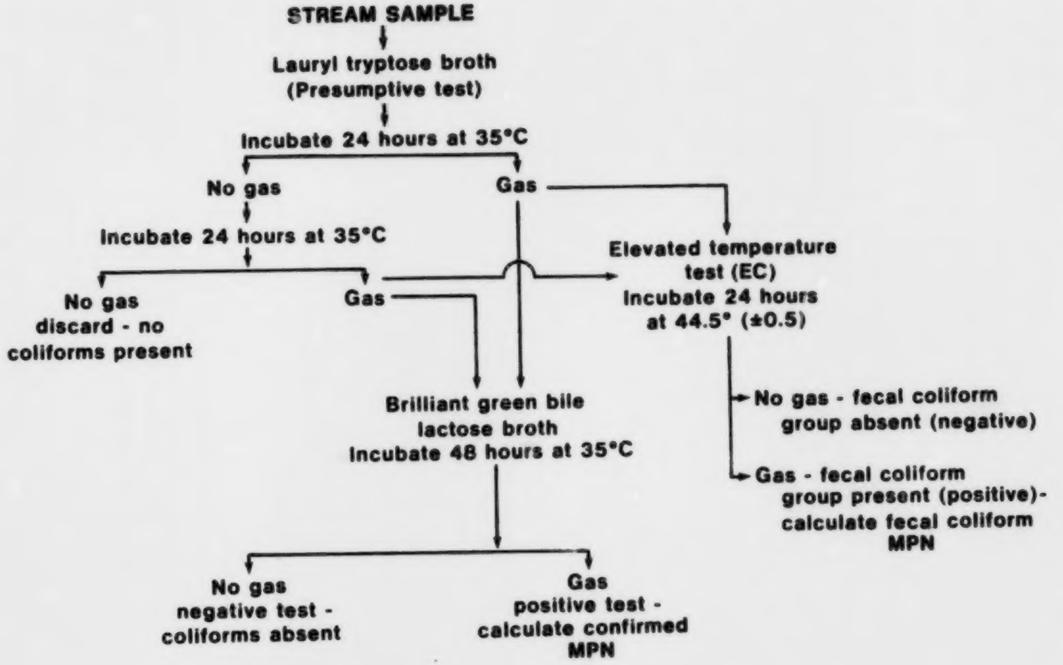
STREAM SAMPLE
Lauryl tryptose broth
(Presumptive test)
Incubate 24 hours at 35°C
No gas
Gas
Incubate 24 hours at 35°C
No gas
discard - no
coliforms present
Gas
Elevated temperature
test (EC)
Incubate 24 hours
at 44.5° (±0.5)
No gas - fecal coliform
group absent (negative)
Gas - fecal coliform
group present (positive)-
calculate fecal coliform
MPN
Brilliant green bile
lactose broth
Incubate 48 hours at 35°C
No gas
negative test -
coliforms absent
Gas
positive test -
calculate confirmed
MPN

Figure 2. Schematic Diagram For Detecting Total Coliform And Fecal Coliform Bacterial Groups

the culture transfer from the presumptive test gas-positive tubes incubated at 35°C to the more selective EC broth for incubation at the elevated temperature.

Heavy growth in the gas-negative EC tubes may be attributed to thermophilic bacteria that respond to the favorable incubation temperature. More frequently, the growth is due to nonfecal coliform organisms transferred from the presumptive medium that were unable to carry out complete fermentation of lactose at the elevated temperature but that attained a sufficient density to show turbidity. In a study of 24,832 coliform strains isolated from various environmental sources, 2,533 (9.8 percent) were able to grow without gas production at the elevated temperature. The occasional manifestation of these anaerogenic (growth without gas production) strains at 44.5°C cannot be related specifically to warm-blooded animals and are not considered part of the fecal coliform group. Extending the incubation of EC cultures beyond 24 hours is not warranted since changes in gas reaction from negative to positive occurred in less than 0.1 percent of cultures examined.

MOST PROBABLE NUMBER CALCULATIONS

A mathematical calculation of the probable density of bacteria in a sample can be made by combining positive and negative results in the multiple tube test. Although most probable number (MPN) calculations can be made from any combination of sample test portions employed, the most frequent multiple tube combinations used are five, replicate, 10-ml portions for potable water examinations and five replicate portions in three-decimal dilutions for base-line data on raw source waters, in water pollution investigations, and when monitoring treated effluent quality. The greater the number of replicates of each sample volume in a dilution series, the greater the test precision. This increase in test precision is illustrated in Figure 3 for MPN values derived from multiple tube tests using 1, 3, 5, or 10 replicate tubes and a test sample containing a true density of 100 coliforms. Obviously, the MPN value is not a precise measurement.

The simplest MPN calculations are those involving potable water tests using five, replicate, 10-ml test portions. When all presumptive tubes in the total coliform test are reported as negative after 48 hours' incubation, the MPN result is stated to be less than 2.2 (< 2.2) total coliforms per 100 ml. If one presumptive positive result confirms in BGLB as a gas positive, the MPN value is 2.2 per 100 ml. Similarly, if two, three, or four confirmatory test results are positive, the MPN value is 5.1, 9.2, or 16.0 total coliforms per 100 ml, respectively. When all five presumptive tubes confirm in BGLB, the MPN value can only be estimated to be greater than 16 total coliforms per 100 ml. The definitive total coliform value can only be determined by a reexamination of the water sample using a five-tube test in three or more decimal dilutions.

With respect to the measurement of stream and marine pollution samples, a five-tube, three-dilution MPN should be used to obtain a broader range of values and a more accurate coliform determination. The practice of using a three-tube, rather than a five-tube, MPN for data gathering to be used in possible enforcement of water quality standards produces a MPN

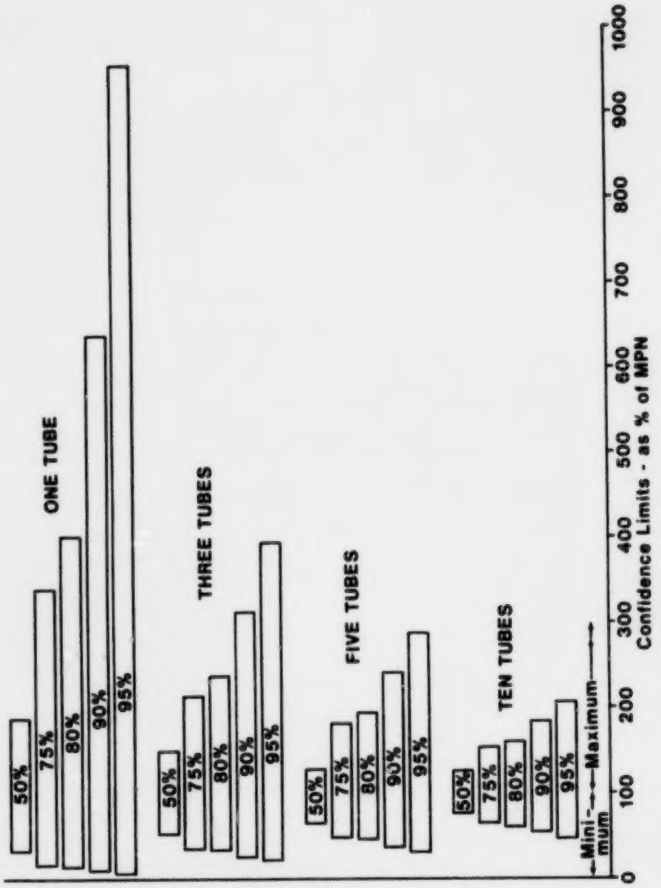

Figure 3. Confidence Limits For MPN Values Derived From Various Numbers Of Tubes In Three-Decimal Dilutions

estimate of significantly reduced precision (Figure 3). The 95 percent confidence limits for a three-tube test range from 21 to 395 percent of the true density whereas the five-tube test results may vary from 31 to 289 percent of the absolute value. As a further point, the tables of most probable numbers were originally calculated to include a positive bias for health safety reasons. Taking this fact into consideration with respect to a three-tube MPN, the reported values may be too high by a factor of 43 percent; whereas, with the five-tube MPN test, the values may be overestimated by only 23 percent (15, 16). For these reasons, a suggested change to the five-tube test would substantially improve the data obtained with little increase in laboratory work or medium cost.

In the five-tube, multiple-dilution-test calculation, the smallest sample volume tested (highest dilution) in which all replicate tubes are gas positive is selected as the starting dilution. The results of this test volume and of the next two smaller volumes are used to determine the positive tube combination. Since the MPN tables are usually limited to values for tests starting with 10-ml sample portions, test results from other starting decimal dilutions require appropriate adjustment based on the following formula:

$$\text{MPN table value} \times \frac{10}{\text{starting dilution}} = \text{MPN per 100 ml}$$

As an example, laboratory results for a sample examined indicated the positive total coliform confirmed results were 5 – 3 – 0 with the smallest sample portion showing all tubes positive being 0.01 ml. With the use of the above formula and the MPN table value for a 5 – 3 – 0 positive tube combination, i.e., 79, the problem is calculated as:

$$79 \times \frac{10}{0.01} = 79 \times 1{,}000 = 79{,}000 \text{ total coliforms/100 ml}$$

Several examples of possible test results are illustrated in Table 7 including the proper selection of positive tube combinations and the calculated MPN value. These examples illustrate the following accepted rules governing proper selection of positive tube combinations:

1. When none of the dilutions used in the multiple tube test have a positive result, the test results are indeterminately low. Thus, if no positive results occur in these three dilutions and the largest sample volume tested was 1 ml, the MPN is reported as <20 per 100 ml. A similar all-negative tube test with a starting dilution of 0.1 ml would be reported as < 200 per 100 ml. Under no circumstances can the construction of a firm MPN value be justified by the assumption that if a larger sample volume had been tested, one or more tubes would have been positive.
2. As a corollary, when all tubes are positive and the starting dilution is 1 ml, the MPN must be reported as > 16,000, or if the starting dilution is 0.1 ml, the MPN value is reported as > 160,000. It is not permissible to assume that if the next larger sample portion had been tested, the results would have produced one or more nega-

TABLE 7. SELECTION OF POSITIVE TUBE COMBINATIONS IN THE MPN CALCULATION

Multiple tube test — positive results per dilution					Selected combination	MPN value per 100 ml
10 ml	1.0 ml	0.1 ml	0.01 ml	0.001 ml		
0	0	0	N.T.*	N.T.	0 – 0 – 0	< 2
0	1	0	N.T.	N.T.	0 – 1 – 0	2
5	3	0	N.T.	N.T.	5 – 3 – 0	79
N.T.	0	0	0	0	0 – 0 – 0	<20
N.T.	3	1	0	0	3 – 1 – 0	110
5	5	0	0	0	5 – 0 – 0	230
N.T.	5	3	0	1	5 – 3 – 1	1,100
N.T.	5	0	2	1	5 – 0 – 3	5,800
N.T.	5	5	5	5	5 – 5 – 5	> 160,000

*N.T. = sample portions not tested

tive results and, thereby, permitted the construction of a firm MPN value.

3. Occasionally, multiple tube results may produce a positive tube skip in the fourth decimal dilution of a sample. For convenience in MPN calculations, this positive tube result must be moved to the third dilution to establish a compatible positive tube combination. Thus, the multiple tube result 5 – 3 – 0 – 1 must be interpreted as 5 – 3 – 1 in establishing the MPN value.

For special studies involving other combinations of replicate tubes and dilutions, a simple approximation of the MPN value may be obtained from use of the following short formula (17):

$$\text{MPN per 100 ml} = \frac{\text{Number of positive tubes} \times 100}{\sqrt{\text{Total sample (ml) in negative tubes} \times \text{total sample (ml) in test}}}$$

For example, when four dilutions of the five-tube test result in a rare positive tube combination such as 5 – 0 – 2 – 1 with the starting dilution of 0.1 ml, the MPN value could be determined from the formula in the following manner:

Number of positive tubes = 5 + 0 + 2 + 1 = 8
Total sample (ml) in negative tubes = 0.0 + 0.05 + 0.003 + 0.0004 = 0.0534
Total sample (ml) in all tubes = 0.5 + 0.05 + 0.005 + 0.0005 = 0.5555

$$\text{MPN per 100 ml} = \frac{8 \times 100}{\sqrt{(0.0534)\,(0.5555)}} = 4600 \text{ (2 significant figures)}$$

If MPN rule 3, previously described, were to be applied to this problem, the value of 5 – 0 – 2 – 1 would be converted to 5 – 0 – 3 and the resulting MPN value would be calculated to be 5,800 per 100 ml. Calculations of MPN's derived by the short formula are more accurate than those derived by the skip accommodation rule, but both numbers are well within the 95 percent confidence limits.

Once the positive tube combinations have been determined, the calculated density can usually be obtained from the appropriate table of MPN values. Tables 8, 9, and 10 are useful for evaluating how proficient the technician is in applying the multiple tube procedure and how adequate the test is for the waters being examined. Such an analysis should be based on a minimum of 50 MPN positive tube combinations derived from laboratory work sheets.

TABLE 8. STATISTICAL EXPECTANCY OF MOST FREQUENT* MPN POSITIVE TUBE COMBINATIONS

Sample size			MPN	95% Confidence zone		Log MPN
10 ml	1 ml	0.1 ml		Low	High	
0	0	0	< 2	—	1.3	—
1	0	0	2	< 0.5	7	0.30103
2	0	0	5	< 0.5	13	0.69897
3	0	0	8	1	19	0.90309
4	0	0	13	3	31	1.11394
5	0	0	23	7	70	1.36173
5	1	0	33	11	93	1.51851
5	2	0	49	17	130	1.69020
5	3	0	79	25	190	1.89763
5	4	0	130	35	300	2.11394
5	5	0	240	68	750	2.38021
5	5	1	348	120	1000	2.54158
5	5	2	542	180	1400	2.73400
5	5	3	918	300	3200	2.96379
5	5	4	1600	640	5800	3.20412
5	5	5	> 1600	1400	—	—

* MPN tube combinations in 67.5 percent samples

TABLE 9. STATISTICAL EXPECTANCY OF FREQUENT* MPN POSITIVE TUBE COMBINATIONS

Sample size			MPN	95% Confidence zone		Log MPN
10ml	1 ml	0.1 ml		Low	High	
1	1	0	4	0.5	11	0.60206
2	1	0	7	1	17	0.84510
3	1	0	11	2	25	1.04139
4	1	0	17	5	46	1.23045
4	2	0	22	7	67	1.34242
5	1	1	46	16	120	1.66276
5	2	1	70	23	170	1.84510
5	3	1	109	31	250	2.03743
5	4	1	172	43	490	2.23553
5	4	2	221	57	700	2.34439

* MPN tube combinations in 23.6 percent samples

These tables include the typical positive tube combinations, the MPN values, 95 percent confidence ranges, and the logarithms of the MPN value that are useful in calculating the geometric mean of a series of MPN results. The most frequent positive tube combinations (67.5 percent of all tests analyzed) are shown in Table 8; those listed in Table 9 have a statistical expectancy of 23.6 percent (18). If more than 7.9 percent of the MPN positive tube combinations recorded are present in Table 10 or consist of more than 1 percent of the improbable codes not listed in any of these groupings, the multiple tube procedure is probably in error. Such an abnormal distribution might result from substances in the water that inhibit bacterial growth, from improper laboratory procedures, or from other causes. Certainly this abnormality indicates the desirability of special investigations to determine the reason(s) for such variation from the expected pattern.

TABLE 10. STATISTICAL EXPECTANCY OF LESS FREQUENT* MPN POSITIVE TUBE COMBINATIONS

Sample size			MPN	95% Confidence zone		Log MPN
10 ml	1 ml	0.1 ml.		Low	High	
0	0	1	2.0	<0.5	7	0.30103
0	1	0	2.0	<0.5	7	0.30103
0	2	0	3.7	0.49	12	0.56820
1	0	1	4.0	0.49	12	0.60206
1	2	0	6.1	1.4	17	0.78533
2	0	1	6.8	1.4	17	0.83251
2	1	1	9.2	2.8	24	0.96379
2	2	0	9.3	2.8	24	0.96848
3	0	1	11	2.8	24	1.04139
3	1	1	14	4.7	35	1.14613
3	2	0	14	4.8	35	1.14613
3	3	0	17	5.0	35	1.23045
4	0	1	17	5.0	36	1.23045
4	1	1	21	6.2	44	1.32222
4	2	1	26	8.6	68	1.41497
4	3	0	27	8.6	69	1.43136
4	3	1	33	11	93	1.51851
4	4	0	34	12	120	1.53148
5	0	1	31	8.8	74	1.49136
5	0	2	43	12	120	1.63347
5	2	2	95	29	240	1.97772
5	3	2	140	48	370	2.14613
5	3	3	180	62	440	2.25527
5	4	3	280	88	750	2.44716
5	4	4	350	89	760	2.54407

* MPN tube combinations in 7.9 percent samples; improbable codes not listed have a theoretically expected 1.0 percent occurrence.

The geometric mean value is applied to data from the analyses of natural water and sewage effluents because this statistical approach is not greatly influenced by the occasional high densities appearing from time to time (19). However, in potable water data analyses, it is essential to recognize those infrequent high values that may occur—reflecting possible intermittent problems in back siphonage or marginal treatment practices (20). The arithmetic mean, unlike the geometric mean and the median, best reflects occasional high values and is the required statistical approach specified in the Federal Drinking Water Standards.

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GUIDELINES ON MULTIPLE TUBE COLIFORM PROCEDURES

Total Coliform Presumptive Test

Potable water: five standard portions, either 10 or 100 ml .... ________
Natural water quality or effluent monitoring: multiple dilutions .... ________
Choice of presumptive test medium ________________________________
Adequate test labeling and tube dilution coding provided .... ________
Sample shaken vigorously immediately before test .... ________
Pipet tip never permitted below 1-inch of sample surface .... ________
Serial dilutions prepared for sample portions of 0.01 ml or less .... ________
Tubes incubated at 35° ± 0.5°C for 24 ± 2 hours .... ________
Examined for gas (any gas bubble positive) .... ________
Twenty-four-hour gas-positive tubes submitted to confirmed test .... ________
Negative tubes returned to incubator .... ________
Examined for gas at 48 ± 3 hours; positives submitted to confirmed test .. ________
Growth extinction MPN calculated from all presumptive tubes with growth ________

Total Coliform Confirmed Test

Presumptive positive tube gently shaken or mixed by rotating .... ________
One loopful or one dip of applicator transferred from presumptive positive tube to BGLB broth .... ________
Incubated at 35° ± 0.5°C; checked at 24 hours for gas production .... ________
Negative tubes reincubated for additional 24 hours; checked for gas production .... ________
Positive tube results recorded; MPN value calculated .... ________

Total Coliform Completed Test

Applied to all positive potable water samples or 20 tests performed each 3 months to reestablish validity of confirmed test .... ________
Applied to all positive confirmed tubes or doubtful colonies on streak plates from each test sample .... ________
Where positive, confirmed tubes are paralleled with a positive EC tube; no further verification in completed procedure needed .... ________
Positive confirmed tubes streaked on EMB or Endo streak plates for colony isolation .... ________
Plates adequately streaked to obtain discrete colonies .... ________
Incubated at 35° ± 0.5°C for 24 ± 2 hours .... ________
Typical nucleated colonies, with or without sheen, given prior selection .. ________
If typical colonies absent or not isolated, atypical colonies selected for completed test identification .... ________
If no colonies or only colorless colonies appeared, the confirmed test for that particular tube is considered negative .... ________
Selected isolated colony chosen for verification was one typical or two atypical to lactose or lauryl tryptose broth and to agar slant for Gram stain ________
Incubated at 35° ± 0.5°C; checked for gas within 48 hours .... ________
Gram stain prepared from 18- to 24-hour-old culture .... ________
Gram negative rods without spores and gas in lactose tube within 48 hours considered positive evidence for coliforms .... ________
Positive tube results recorded; MPN value calculated .... ________

Fecal Coliform Test

Applied as an EC broth confirmation of all positive presumptive tubes .... ________
EC tubes placed in water bath within 30 minutes of transfer .... ________
Incubated at 44.5° ± 0.2°C for 24 hours .... ________
Gas production considered positive test for fecal coliforms .... ________
Positive tube results recorded; MPN value calculated .... ________

Most Probable Number Calculations

Smallest test portion with all tubes positive selected as starting dilution . . . ________
Positive tube codes properly adjusted to accommodate skip results . . . . ________
MPN table values adjusted to reflect starting sample dilution . . . . ________
MPN short formula used to calculate unusual multiple tube combinations . ________
Analysis of positive tube results indicated normal distribution
of possible codes . . . . ________

CHAPTER VIII
MEMBRANE FILTER COLIFORM PROCEDURES

The membrane filter (MF) procedure for the enumeration of total coliforms was introduced into *Standard Methods* as a tentative method in 1955 (1) and established as both a standard test and an alternate to the multiple tube procedure in 1960 with the publication of the 11th edition of *Standard Methods* (2). The basic procedure involves filtering a known volume of water sample through a MF of optimum pore size for full bacterial retention. As the water passes through the pores, bacteria are entrapped on the upper surface of the MF. The MF is then placed in contact with either a paper pad saturated with liquid medium or directly over an agar medium to provide nutrients for bacterial growth. Following incubation under prescribed conditions of time, temperature, and humidity, the cultures are examined for coliform colonies that are then counted and recorded as a density of coliforms per 100 ml of water sample.

MEMBRANE FILTER TEST LIMITATIONS

The majority of water samples can be tested by MF methods. Some types of samples, however, cannot be filtered because of turbidity (3), excessively high noncoliform bacterial populations (3), or heavy metal compounds (4,5). These difficulties may be encountered in examining samples from some well waters, impounded reservoirs, small lakes, industrial effluents, and poor quality chlorinated effluents (6,7).

The presence of suspended material in the sample may limit any application of the MF procedure. This limitation will depend on the volume of sample filtered, the type of suspended material, and the thickness of the layer of suspended material on the filter surface during incubation of the sample. Relatively thin layers of gelatinous, finely divided, or hygroscopic materials, such as suspended iron, manganese, alum flocs, or algae, may clog the pores of the filter or may cause a spreading film of growth during incubation. Thicker surface layers of crystalline or siliceous materials may cause little or no difficulty. Where the coliform density is known to be so high that the sample volume need not exceed 2 or 3 ml, there is little chance that turbidity on the filter will cause problems. However, if few coliforms are present and the sample has obvious turbidity, then the multiple tube procedure should be used.

Large populations of noncoliform organisms in potable water supplies may make coliform analysis difficult by either the multiple tube (MPN) or the MF procedure, because of possible suppression of coliform detection. The occurrence of high-density, noncoliform populations is particularly evident on the MF where overwhelming numbers of bacteria may counteract the suppressive mechanism of M-Endo medium and produce a massive overgrowth that masks visual detection of coliform colonies on

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the filter surface. Since the selective mechanism of M-Endo MF medium cannot cope with poor quality potable waters containing in excess of 400 noncoliform to one coliform per 100 ml, the five-tube MPN procedure must be used for coliform analysis. The preferred solution to this problem is not to use the less precise MPN procedure but maintain a free-chlorine residual in the potable water supply that protects against contamination and controls the general bacterial population.

Some waters, highly polluted with industrial wastes, have been found to contain more than 1 ppm of zinc or copper. Apparently metallic ions that exert a bactericidal or bacteriostatic effect can be adsorbed on the membrane and, thus, prevent bacterial growth. Samples from such waters should be collected in sample bottles containing a chelating agent. In addition, the use of a 2-hour MF culture enrichment before planting the membranes on selective media or in more selective incubation temperatures may be needed for optimum recovery of stressed coliform organisms.

Sewage that has received only primary treatment followed by chlorination or other sewages containing phenols or toxic metals from industrial wastes cannot be examined by the MF procedure. Samples of chlorinated primary effluent often exhibit temporarily reduced coliform density (1,000 organisms or less per 100 ml), and 4 to 6 ml of sample are needed to obtain representative coliform density measurement. The upper limit for the amount of primary effluent that can be filtered, however, appears to be 1 ml; with larger volumes, extraneous materials clog the MF pores, deposits build up over the effective filtration area, growth of discrete coliform colonies is prevented, and the resulting culture confluency makes selective counting of coliform colonies difficult, if not impossible. Therefore, wastewaters of this character must be examined by the multiple tube procedure, and it must be realized that a significant number of false-positive results may occur in the confirmed MPN on chlorinated primary effluents, particularly when stormwater runoff enters the mixed sewage collection.

When sewage receives secondary treatment, a MF limitation related to pore clogging does not exist because there is little gelatinous material or microfecal pellets remaining in this higher quality effluent. However, the effect of disinfection action on residual coliforms in those secondary effluents receiving a chlorination treatment does limit MF procedures to the two-step (pre-enrichment) procedure for total coliforms. Apparently the 2-hour enrichment is necessary to permit organisms sufficient time to achieve repair of damaged enzyme systems before contact with the selective Endo medium. Recent data (8) indicate that a direct application of the MF fecal coliform procedure to chlorinated sewage effluents may recover fewer of these organisms than does the multiple tube procedure. In this instance, the critical factor is temperature acclimation for the stressed coliforms surviving disinfection exposure (9).

Review of the attenuated fecal coliform recovery problem suggests that chlorine inactivation of some coliform cells might be reversed provided enrichment (10,11) and temperature acclimation (11,12) were possible without compromising the specificity of the test. All enrichment procedures previously developed for the membrane filter technique required a manual transfer of the membrane filter cultures from one membrane to another. Recognizing media manipulations are time consuming in the laboratory, a new approach (13) incorporating a two-layer-enrichment (lactose broth + 1.5 percent agar) differential growth medium (M-FC broth + 1.5 percent agar) allows for repair and subsequent reproduction of those fecal coliforms that have been stressed by exposure to chlorine, industrial wastes, or marine waters.

A two-layer medium (Figure 4) is prepared by dispensing approximately 5 ml of M-FC agar into each culture dish (50- × 12-mm), permitting the agar to solidify, then adding 2 ml of normal strength lactose broth in 1.5 percent agar over the M-FC agar. Since the ingredients of the two agar layers will eventually diffuse into each other, it is suggested that the base M-FC agar be prepared in advance and the lactose agar overlay added 1 hour before using.

After the MF is placed on the two-layer medium, the plates are incubated at 35°C for 2 hours after which the temperature is increased to 44.5°C for 22 to 24 hours to attain the necessary selectivity. All blue colonies are counted with the aid of a binocular scope employing 10 to 15× magnification and a fluorescent light source. Verification of fecal coliforms isolated on the test medium is performed by subculturing each blue colony into either phenol red lactose broth or lauryl tryptose broth for 24 to 48 hours at 35°C. Tubes showing gas production within this period are subcultured to EC broth and incubated in a water bath for 24 hours at 44.5°C ± 0.2°C.

The decision to use the slightly more involved two-layered medium procedure in preference to the direct M-FC method should be based on a demonstration of increased verified recovery of fecal coliforms from samples routinely examined. MF's with 2.4-micron surface-opening diameters (HC type) may also improve recovery of the direct M-FC method.

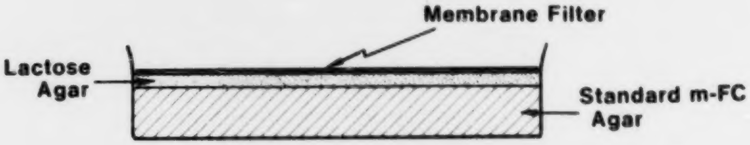

Figure 4
Two-Layered m-FC Agar

EVALUATION OF THE MF FECAL COLIFORM TEST FOR SEWAGE EFFLUENTS

Any decision to use the layered M-FC agar procedure or any subsequently proposed fecal coliform MF procedures in the bacterial quality assessment of chlorinated sewage effluents must be based on laboratory data that demonstrate at least an 80 percent agreement between parallel MF and MPN fecal coliform methods. Approximately 100 samples chosen from a variety of sewage plant effluents should be used

used in this MF-MPN comparative study. Erratic data from both the MF and MPN procedures may be caused by fecal micropellets in poorly treated sewage effluents. Sample blending for 6 to 30 seconds at 3,000 rpm does help alleviate this problem. When chlorinated secondary effluents are examined from plants subject to wide variations in effluent quality because of seasonal or other factors, it is suggested that every fifth sample be examined by both the MF and the five-tube procedure until comparability of results is verified. The same approach should be used on combined sanitary sewer-stormwater overflow samples collected during early runoff periods when the turbidity is high. This approach should be continued until the reproducibility of results by the MF procedure is established.

EVALUATION OF THE MF TOTAL COLIFORM TEST FOR POTABLE WATERS

Initial comparison of the MF test and the multiple-tube MPN completed test procedure by laboratory parallel testing is recommended. Such an evaluation establishes the expected sensitivity of the MF test to the analyzed waters from a given geographical area and also permits the technician to gain necessary experience in the use of the MF technique.

In such an evaluation, the completed test rather than the confirmed test should be used to ensure the validity of the coliform results used for reference. The confirmed test is not a perfect screening procedure for coliform bacteria since it may yield a positive reaction in the absence of the coliform group, i.e., false-positive test. Coliform MPN results from the examination of soils (14) and various waters (15) demonstrate that significant differences in coliform numbers can occur between the confirmed and completed tests. The bacterial flora of a given water, the age of the sample, or the suppressive action of the brilliant green dye and bile salts in the confirmatory medium can contribute to the possible occurrence of such differences (16).

The comparative evaluation should extend over a 3-month period (minimum) and include a variety of municipal water samples, wells, cisterns, lakes, and raw source waters at public water intakes.

Data from both the MF and completed test procedures should yield the same information about the sanitary quality of water examined. When potable water samples are examined to evaluate these procedures, a sample is defined as unsatisfactory if four or more coliforms are detected per 100 ml by the MF test or three or more positive confirmed tubes are observed in the MPN procedure. Thus, the comparison for equivalency does not require that the two test procedures demonstrate numerically equal coliform densities. In instances where only raw source water samples are used in the evaluation, 80 percent of the MF values should be within the 95 percent confidence limits of the MPN completed test results. Multiple tube results are higher numerically than MF results because MPN numbers represent a statistical estimate of the true density in the sample, with the five-tube MPN table of values including a 23 percent positive bias as a safety factor (17). Additional information may be obtained from numerous comparisons of the MPN and the MF procedures used with potable water, natural fresh waters, sewage, and marine waters (18-35).

TOTAL COLIFORM MF PROCEDURE

Successful application of MF methods requires development of good laboratory and routine operational practice (36). Preliminary activities include: recording sample data in the laboratory log; disinfection of laboratory bench-top working area; and assembly of the necessary sterile filtration equipment and sterile materials (MF's, culture containers, pipets, graduated cylinders, dilution blanks and medium).

To prepare Petri dishes for the MF, place one sterile absorbent pad in each culture dish (using sterile forceps), unless an agar medium is being used. The amount of culture broth necessary to saturate an absorbent pad varies as a result of pad thickness and degree of dryness—from 1.8 to 2.2 ml. Pour any excess medium from the culture dish before rolling the membrane over the absorbent pad. If the excess is not removed, flooding of the membrane may occur and cause confluent growth on the membrane. Insufficient medium results in small "starved" colony development. When agar medium is employed, dispense 3 to 4 ml of the melted agar medium directly to each culture dish.

The filtration assembly should be sterile at the beginning of each filtration series that may involve 30 or more samples. A filtration series is considered interrupted if there is an interval of 30 minutes or longer between sample filtrations. Resuming filtration after such an interruption requires another set of sterile filtration units and is considered a new filtration series. This protocol minimizes chance contamination of funnels from spills and protects filter holders from leakage of contaminated waters during filtration malfunctions. Rapid resterilization of the funnel (see Sterilization Procedures; MF Filtration Equipment in Chapter V) by UV, flowing steam, or boiling water may be practiced between sample filtrations at the bench.

A standard sample volume of 100 ml must be analyzed for all public water supplies, e.g., treated water supplies. In potable water, test results should most frequently indicate no coliform detection in 100-ml volumes, although rare occurrences of one to three coliforms are permissible provided the arithmetic mean coliform density for a given supply remains below one coliform per 100 ml. The coliform content of treated water supplies must be less than one total coliform per 100 ml as measured by the MF procedure. Untreated water supplies (individual wells, springs, etc.) may have excessive noncoliform bacterial populations that will necessitate examining two 50-ml portions per sample.

All potable water sample volumes must be measured within a ± 2.5 percent tolerance as specified in the MF procedure since this test is quantitative. When glass filter funnels are used, the 100-ml gradation may be used after its accuracy has been verified. Although metal funnels may not have 100-ml marks impressed on the interior surface, use a waterproof, heat-resistant ink or enamel to inscribe a line at the 100-ml water level.

For the most accurate measurement of potable water sample volumes, use graduated cylinders. An individual, sterile, graduated cylinder or volumetric pipet should be assigned to each sample examined in the filtration series. Sample volumes can then be measured, poured into the funnel, and filtered. A small portion of sterile buffered dilution water (approximately 25 ml) is flushed into the graduated cylinder for rinsing and then poured into the MF funnel being used for that sample. Follow this procedure with two separate short rinses (with approximately 20 to 30 ml of sterile dilution water) to flush any residual bacteria from the funnel walls onto the MF surface. Rinsing the graduated cylinder and funnel before removing the MF not only ensures transfer of all bacteria in the sample to the membrane surface but also prevents carryover of coliforms to the next sample.

MF's are fragile and may be easily damaged by improper handling. Grasp the outer part of the MF, outside the effective filtering area, with sterile, smooth-tipped forceps. This procedure avoids smearing entrapped bacteria or the possibility of piercing the MF surface and breaking its retention capabilities. Place the sterile MF on the filter holder, grid-side up, centered over the porous part of the filter support plate. To avoid damage to the MF, the funnel should not be turned or twisted while it is being seated and locked to the lower element of the filter holder. Filter holding units featuring a bayonet-joint and locking ring to join the upper element to the lower element require special care on the part of the operator. Turn this locking ring sufficiently to give a snug fit, but do not tighten excessively.

Immediately before filtering a measured sample, invert the sample and shake it vigorously. This vigorous shaking is needed to obtain a homogeneous distribution of suspended bacteria and is of particular concern with turbidity-laden waters. Turbidity in water settles rapidly, pulls suspended bacteria into the bottom sediment, and thereby creates an uneven distribution of the bacterial population in measured aliquots.

After shaking the sample thoroughly, pour or pipette the measured sample volume into the funnel with the vacuum supply line connection turned off. To avoid uneven distribution of organisms over the effective filtering area, the vacuum should never be applied simultaneously with the addition of the sample test portion. Before dispensing 10 ml or less, add approximately 10 ml of sterile dilution water to the funnel to ensure uniform dispersion of the bacterial suspension. Then apply the vacuum to force rapid passage of the sample through the MF, after which, rinse the funnel wall with 20 to 30 ml of sterile dilution water. After the first rinse has passed through the filter, repeat this rinsing procedure. Extensive tests have shown that with proper rinsing technique, bacterial retention by the funnel walls is negligible.

The buffered dilution water that is used for rinse water in the MF procedure is often prepared in large flasks or carboys, autoclaved, and stored in the laboratory until needed. Since these containers may vary from 1-liter flasks to 20-liter carboys, the rinse water is generally dispensed by siphoning through glass, Teflon, or rubber tubing to the MF funnels or is poured into smaller, sterile wash bottles for ease in handling. Caution must be exercised that the siphoning devices and dispensing wash bottles do not become contaminated and, thereby, contribute microbial contamination to the filtration procedure. A single occurrence of heavy microbial growth in the rinse water can nullify the results of an entire day's water testing program by completely "masking" the membrane with noncoliform growths that interfer with coliform colony or sheen development. Using a fresh sterile rinse water supply and dispensing system each day will avoid this contamination problem.

To ensure that no contamination exists at the start of testing, a sterile 100-ml dilution blank should be subjected to the MF procedure, before the initial processing of water samples. Whenever possible, analyze potable water samples first in a filtration series, followed by natural waters, then sewage and industrial effluents. Inject a sterile test water sample at the conclusion of each grouping of waters and effluents and one at the conclusion of the filtration series. The purpose of this quality control procedure is to ensure materials were sterile at the start of filtration and to isolate possible cross-contamination if the technician fails to adequately rinse all organisms onto the filter surface of a polluted sample. When sterile controls indicate contamination occurred, all data on samples affected should be rejected and a request made for immediate resampling of those waters involved in the laboratory error.

Upon completion of the rinse procedure, turn off the vacuum supply to the filtration assembly to avoid accidentally tearing the filter while transferring it in the next step. Disengage the filtration assembly and carefully transfer the MF, using sterile forceps, to a Petri dish containing a medium-saturated absorbent pad or an agar preparation. Proper contact between the MF and the absorbent pad or agar substrate requires the underside of the membrane to be uniformly wetted with culture medium without air bubble entrapment.

Air bubbles trapped between the membrane and the substrate medium can easily be recognized on M-Endo MF medium as colorless or light pink spots on the membrane or can be seen through the agar layer in the inverted culture dish. The entrapment of air bubbles must be avoided in the interfacing of the effective filtration area of the MF with the substrate because this condition becomes an immediate barrier to bacterial contact with the nutritive substrate. Air bubbles are produced when membranes are rolled too rapidly over the substrate, engulfing air pockets. Other causes of air bubble entrapment may relate to changes in agar surface from desiccation during storage or foaming of agar during the rapid ejection of medium from an automatic syringe or pipet into culture dishes. These entrapments of air block the diffusion of nutrients from either the medium saturated absorbent pad or agar preparation to any bacteria on the MF surface directly above. This condition results in diminished potential for growth of the viable bacterial cells into differentiated colonies or hastens their death through desiccation. The net result would be an occassional reduction in the detection of low levels of coliforms in potable waters. Therefore, inspect all MF cultures before incubation for any air bubble entrapment inside the effective filtration area. Air bubbles are easily removed by simply lifting the membrane with sterile forceps and rerolling it onto the medium saturated pad or agar substrate. Thereupon, close the culture container, invert it, and promptly place it in the appropriate incubator, preferably within 10 to 15 minutes after filtration.

INCUBATION OF MF CULTURES

MF examinations for total coliform recovery require a 22- to 24-hour incubation period at 35°C for optimum growth and sheen development.

This time period is especially important when examining potable water samples since incomplete disinfection may have created stressed coliforms with damaged metabolic pathways. These coliforms are initially slow to develop the normal lactose fermentation end products that are the basis for differentiation.

The incubator should maintain a high level of humidity (approximately 90 percent). Reduced humidity often permits the surface of the membranes to lose moisture more rapidly than it is replenished by the diffusion of medium from an agar or absorbent pad substrate. As a result, growth failure or, at best, small or poorly differentiated colonies may result. A conventional, hot-air incubator may be used; however, cultures in loose fitting Petri dishes must be placed in a tightly closed container, along with wet paper or cloth to maintain the necessary humid atmosphere. A vegetable crisper, such as is used in most home refrigerators, is satisfactory for this purpose. Tight-fitting plastic Petri dishes are preferred because the required humidity is established for each culture by the evaporation of some of the medium within the confines of the individual dish. No modification for higher humidity in the air incubator is necessary when tight-fitting plastic culture dishes are used.

MF COLONY COUNTING

Coliform colonies are best counted while in the moist state associated with their growth. Magnification of 10 to 15 diameters and a daylight fluorescent light source adjusted to an angle of 60° to 80° above the colonies are essential for optimum reflection of the golden metallic luster from coliform colonies on an Endo-type medium. The procedure of drying MF cultures to improve sheen visibility before counting is open to criticism whenever such colonies are to be subjected to the coliform verification procedure. Colonies exposed to more than a few minutes of drying may not be capable of growth following transfer to lactose broth tubes. Comparisons made by different technicians in several laboratories indicate there is no significant advantage for this time-consuming drying procedure. The use of the recommended fluorescent light source positioned above the MF culture will yield excellent reflection of the metallic luster from coliform colonies.

The typical coliform colony has a pink to dark red color with a metallic surface sheen. The sheen area may vary from a small pin-head size to complete coverage of the colony surface. All members of the coliform group grow and develop a metallic sheen on Endo-type media. Development of colonies of noncoliform bacteria is generally restricted by the medium, but there are exceptions for certain waters where noncoliform growth may cover the filter surface.

Noncoliform colonies vary in appearance from colorless to a deep red color. Colonies having a red color and a "small flake" or "speck" of shiny material resembling a metallic sheen are the most confusing of the noncoliform types. The novice has great difficulty with confluent colonies, with mirror reflections of fluorescent tubes, which are confused with sheen, and with water condensate and particulate matter, which are occasionally mistaken for colonies. Thus, there is a tendency for the novice to err on the high side in MF counts. Technicians who have not attained proficiency in coliform colony recognition should transfer doubtful colonies to lactose (or lauryl tryptose) broth tubes for verification as coliform organisms.

SELECTION OF COUNTABLE MEMBRANES

Always report total coliform densities determined by the MF procedure as "total coliforms per 100 ml" regardless of the size of test portion used or the nature of the sample (potable or polluted water). The coliform density may be calculated from one or more MF counts resulting from testing serial sample portions, provided the counts are in the 20 to 80 colony range and the total count of all colonies on the MF does not exceed 200. When the filter counts are less than 20, total all test results for the sample and relate the coliform density to the total volume filtered, using the following equation:

$$\text{Total coliform colonies per 100 ml} = \frac{\text{coliform colonies counted} \times 100}{\text{volume of sample filtered}}$$

Ideally membranes selected for counting total coliform populations in polluted waters should have from 20 to 80 coliform colonies and not exceed 200 total bacterial colonies. If different volumes of sample are examined, it is permissible to total the counts on each membrane and base the value on the total volume of sample examined. For example, if duplicate 50-ml portions are examined and the two membranes contain 5 and 3 coliform colonies, respectively, the count should be reported as 8 per 100 ml. This count is reliable since a 100 ml sample portion actually was examined. Similarly, if 50-, 25-, and 10-ml portions were examined and the counts were 15, 6, and < 1 coliform colonies, respectively, the 15 and 6 would be totaled and the count, based on a 75-ml volume, would be calculated using the above equation and reported as 28/100 ml. If 10-, 1.0-, and 0.1-ml portions were examined and counts were 40, 9, and < 1 coliform colonies, respectively, the result would be reported as 400 per 100 ml. Considering the last example, if the 10-ml portion has a total coliform count of 40 but the total bacterial colony count (coliform plus noncoliform colonies) is greater than 200, the total coliform count would be reported as > 400 per 100 ml. Subsequent samples from this source water would require adjustment of the sample volume examined by the MF procedure to obtain specific and reliable counts.

INTERPRETATION OF MF CULTURES

Sample portions with an extremely high density of coliform colonies (greater than 80 colonies per MF) should be reported as greater than the number of coliform colonies actually counted. Membranes showing a mass of growth, devoid of defined colonies, should be reported as "confluent growth" even if sheen covers the entire mass of growth. In both instances, another sample should be collected and adjustments should be made regarding sample volume examined.

Drinking water acceptance for public consumption requires demonstration of minimal numbers of coliform organisms in individual samples and at a limited frequency in all samples examined per month as set forth in EPA's *Primary Drinking Water Standards* (37). Where total coliform density is determined by a multiple tube procedure that analyzes 50 ml of sample divided into 5 portions of 10 ml each, the monthly report of bacteriological quality is based on a 5 percent limit of samples having 3 or more tubes positive. By contrast, the MF technique involves a direct count of coliforms per 100 ml. When the MF technique is used, the bacteriological rating of a public water supply is based on two qualifications—coliform density not to exceed an arithmetic mean of one per 100 ml for all samples examined per month and a limit on unsatisfactory samples (5 or more coliforms per 100 ml) in one sample when less than 20 per month are examined or in 5 percent of all samples when more than 20 are examined each month. Because the range of accurate total coliform values can be from less than 1 to 80 organisms per 100 ml, individual densities may make the monthly arithmetic mean limit of 1 total coliform per 100 ml difficult to obtain without a significant increase in the number of routine samples. This position stimulates the more desirable reaction of intensifying the monthly sampling frequency. When using the multiple tube procedure, however, the percent of positive tubes per month may easily be lowered with only a slight increase in sampling frequency since each test adds five individual tubes to the total base number used in monthly calculation. Thus, the desired increase in sample frequency is partially lost on those water supplies using the multiple tube test. To offset this MF-MPN inequality, interpretation of the regulations should recognize that even though a single MF total coliform result may prevent the arithmetic mean attainment of one coliform per 100 ml limit, the water quality is still classed as satisfactory because the frequency of this unsatisfactory MF coliform occurrence has not exceeded the *Primary Drinking Water Standards*.

Various types of water supplies are used for drinking water throughout the United States. Because some are untreated (usually private supplies) and others are ineffectively treated, results obtained by the MF procedure can range from "no growth" to "confluent growth" per 100 ml. The following guidelines are recommended for reporting MF procedure results:

Confluent growth—no discrete colonies, growth covering the entire filtration area of the membrane. Results should be reported as "confluent growth." The water supply should be treated before additional examination.

TNTC (too numerous to count)—The total number of bacterial colonies (coliform plus noncoliform) are too numerous or not sufficiently distinct to obtain an accurate count, or both; usually greater than 200 colonies per membrane. It is permissible to adjust the individual sample volumes filtered; however, the total sample volume examined must equal 100 ml. For example, rather than examining a 100-ml portion, examine two 50-ml portions or four 25-ml portions. Coliform colonies observed on each membrane are then totaled and reported per 100 ml.

If the 100-ml portion examined was found to contain colonies too numerous to count (TNTC) but distinct, typical coliform colonies are observed and the number of coliform colonies is:

- less than 4 per 100 ml—then the report should *not* indicate the sample *to be satisfactory* since a high density of noncoliform organisms may inhibit growth or sheen development or both of the coliform colonies. Treatment should be recommended before another sample is collected for examination.

- greater than four per 100 ml—then the report should indicate the sample to be unsatisfactory. The number of colonies should be reported as greater than the number counted in the estimate. The water supply should be treated and another sample collected for examination.

Examination of some water supplies, usually private supplies, may result in greater than 200 noncoliform colonies per membrane when a 100-ml sample is examined. Such occurrences emphasize a need for effective treatment of the water supply before it is resampled and examined. Employment of an MPN procedure to examine such potentially hazardous water supplies should be discouraged since changes in methodology will not improve water quality.

VERIFICATION OF COLIFORM COLONIES

When coliforms are found in potable water samples, initiate a rapid alert to the proper authorities and a request for repeat sampling at the same sites on the distribution network. Retain these cultures, however, until subjected to the verification procedure since synergistic false-positive coliform reactions on Endo media may occur (38,39). This supplemental procedure consists of transferring each coliform colony to lactose or lauryl tryptose (LTB) broth and then to BGLB for evidence of gas production at 35°C within the 48-hour limit. If all coliform-type colonies cannot be transferred, verify a random selection of at least 10 sheen colonies. Avoid direct transfer of colonies to BGLB because of the inherent lower recovery of stressed coliform strains in this more selective medium. Omitting the BGLB step is undesirable since this medium eliminates some of the false-positive results from the lactose or LTB broth.

In an effort to expedite the time delay resulting from verification of sheen colonies, it is permissible to transfer growth from each colony into pairs of lactose or LTB broth and BGLB broth tubes. In this procedure, the verification is completed in 24 hours if both the lactose or LTB broth culture and the BGLB broth culture produce gas at 35°C. However, in those instances where the pair of cultures is negative, the lactose or LTB broth culture is reincubated for the second 24-hour period, and if then positive, a confirmation into a new tube of BGLB is necessary before verification is complete. This procedure of double inoculation from each sheen colony could reduce the test time from 80 to 90 percent for all coliform colony verification.

From the number of BGLB cultures that produce gas within 48 hours at 35°C, calculate the percent of colonies verified as coliforms. Then use this percent figure to adjust the reported coliform count per 100 ml. As an example, 10 sheen colonies from one culture might be verified through inoculations into LTB broth then to BGLB; however, only 8 tubes produce gas in BGLB. The percent verification is 80. The original coliform count was recorded as 20 organisms per 100 ml. Based on the verification of a random selection of 10 such colonies, the final coliform count recorded and reported would be 16 (80% × 20) organisms per 100 ml.

Verification of coliform occurrences in potable water or in at least one set of critical stream pollution samples obtained during a field survey is important for several reasons. This additional procedure provides useful reinforcement of the laboratory findings in any legal action involving records subpoenaed for court use and in decisions pertaining to reclassification of interstate carrier water supply systems. The verification procedure is also an essential part of technician self-training in accurately discerning coliforms, particularly on those MF cultures that exhibit poor sheen development because of sample turbidity and spreading films of bacterial growth. The inexperienced technician frequently finds the deep red colonies difficult to classify, especially where the presence or absence of a metallic sheen is the only distinguishing characteristic. In some instances the true colony sheen has been confused with mirror reflection of fluorescent microscope lamp tubes on the moist shiny surface of pink or red colonies. This confusion is greatest with dark red colonies with granulated surfaces that reflect diffused light similar to that of a sheen colony. Water condensate droplets and turbidity particles combined with this mirror reflection have also frequently been classified as coliform bacteria by the novice technician. This problem of proper coliform discernment by a new technician is solved only by actual practice and experience in counting colonies, supported by the verification procedure.

CHOICE OF TOTAL COLIFORM METHODS

The bacteriologist has a choice of methods for the detection of total coliforms by the MF procedure. Either M-Endo MF broth or LES Endo agar may be used in a single step procedure, i.e., after sample filtration, the MF culture is incubated solely on one of these two Endo-type media. As an alternative, after sample filteration, first incubate the MF culture 1.5 to 2 hours at 35°C on lauryl tryptose broth for enrichment. Then transfer the MF to a new absorbent pad saturated with M-Endo MF broth, or to the bottom of the same culture dish containing LES Endo agar for incubation at 35°C for 20 to 22 hours to differentiate coliform colonies. With several options for medium choice, with or without enrichment, laboratory personnel have the opportunity to evaluate these methods for optimum coliform recovery from waters in their geographical area. Such an evaluation should include a variety of samples and also a verification of a proportion (not less than 10 percent) of the sheen colonies. Enrichment may be beneficial for the optimum recovery of attenuated coliforms from chlorinated effluents of secondary sewage treatment plants and industrial wastes containing significant concentrations of heavy metal ions.

FECAL COLIFORM MF PROCEDURE

For those laboratories with the technical capability to perform the MF procedure for fecal coliform enumeration, the only special items necessary are a water bath, which can be regulated at 44.5°C ± 0.2°C, M-FC broth, and sealable plastic bags to protect the cultures while immersed in the water bath incubator. For specific details on the MF procedure, follow the recommendations described for total coliform MF tests in the preceding portion of this chapter.

Examination of natural bathing water, stormwater runoff, raw sewage, and treated sewage effluents for fecal coliforms requires a range of test volumes to obtain suitable fecal coliform densities on the membrane within the range of 20 to 60 colonies. When the bacterial level of the sample is totally unknown, it is necessary to filter several decimal quantities of sample to obtain a countable density of coliforms. The best method is to estimate the ideal quantity expected to yield a countable membrane and use two additional quantities representing one-tenth and ten times that quantity. Use the best density within the 20 to 60 fecal coliform colony range for the fecal coliform colony count and disregard the remaining two membrane culture results.

This procedure parallels the field survey practice of inoculating four or five decimal dilutions of polluted water samples in the multiple tube procedure and then choosing the three consecutive decimal dilutions that give an approximately even split of negative and positive tubes for use in calculating the MPN value. Data in Table 11 are a guide for selecting the appropriate volume of various waters and wastes. Using peptone dilution water may increase recovery of stressed cells.

TABLE 11. SUGGESTED GUIDE FOR FECAL COLIFORM FILTRATION QUANTITIES

Water source	Quantities filtered (ml)						
	100	50	10	1	0.1	0.01	0.001
Lakes, reservoirs	X	X					
Wells, springs	X	X	X				
Water supply, surface intake		X	X	X			
Natural bathing waters		X	X	X			
Sewage treatment plant secondary effluent			X	X	X		
Farm ponds, rivers				X	X	X	
Stormwater runoff				X	X	X	
Raw municipal sewage					X	X	X
Feedlot runoff					X	X	X

Following sample filtration, place the MF on an absorbent pad saturated with M-FC broth (or the same medium prepared in 1.5 percent agar) contained in Petri dishes with tight fitting lids. After inspecting for air bubbles that must be released from between the medium and the membrane, insert the cultures into sealable plastic bags. These waterproof plastic bags (Whirl-Pak or equivalent) may be used to hold five to eight culture dishes during submersion. These cultures must be placed in the incubator within 30 minutes of filtration since the elevated temperature is critical to the fecal coliform test selectivity. Incubate at 44.5°C for 24 ± 2 hours, then examine the MF cultures under low-power magnification for fecal coliform (blue colony) occurrences. Count and calculate fecal coliform density per 100 ml. Verify colonies using LTB (35°) then EC (44.5°).

Occasionally gray to cream-colored colonies may be observed on M-FC cultures. These organisms are not fecal coliforms and should not be counted as such. Count M-FC cultures promptly after their removal from the incubator since exposure to room temperature for more than 30 minutes may permit some of the gray to cream-colored nonfecal coliform colonies to ferment enough lactose to develop a pale blue color.

MF REPLICATES FOR SPECIAL STUDIES

The generally accepted practice of filtering a single 100-ml portion of potable water and single portions of three different increments of a polluted water is not acceptable in standard plate count determinations or in special research studies. In these latter instances, where precision is most demanding, replication of filtration volumes is essential. Some idea of the importance of replication to improve test precision can be seen from a study of Figure 5. This bar graph was developed from data obtained from 9 different samples examined by the nutrient agar pour plate technique and from 25 different samples examined by the MF

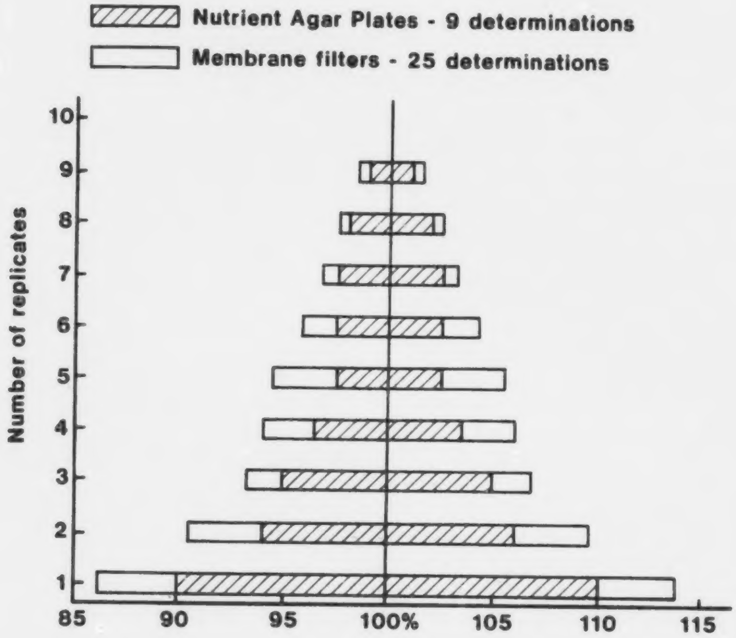

Figure 5. Relative Precision of Replicates for Pour Plate and Membrane Filter Cultures

procedure using a similar enrichment agar. By increasing the number of replicates, the 95 percent probability for the averaged MF or pour plate count to approach the true density became significantly greater. Substantial improvement in levels of data precision appears when 3, 5, or 10 replicates are selected, and increasing the number of replicates should be carefully considered. Thus for routine analyses, every tenth sample should be done in duplicate to verify continued level of data precision.

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GUIDELINES ON MEMBRANE FILTER COLIFORM PROCEDURES

Membrane Filter Test Limitations

Laboratory recognized that turbid waters, excessive noncoliform populations, heavy metal ions, and poor quality chlorinated effluents limits application ________

Application in the Standard Test

Demonstrated, by an initial parallel testing, that the MF yields essentially the same information as the completed test MPN on a variety of waters ________

Total Coliform Membrane Filter Procedure

Filter funnel and receptacle sterile at start of series .... ________
Funnel rapidly resterilized by UV, flowing steam, or boiling water ...... ________
Absorbent pads saturated with medium; excess discarded .... ________
Test portions measured not less than 100 ml (± 2.5 percent) or divided for multiple filtration of potable water .... ________
Funnel rinsed by flushing several 20- to 30-ml portions of sterile buffered water through MF .... ________
MF removed with a sterile forceps grasping the area outside the effective filtering area .... ________
MF rolled over medium pad or agar so air bubbles not formed .... ________

Incubation of Membrane Filter Cultures

Total incubation time 22 to 24 hours at 35°C (± 0.5°C) .... ________
Incubated in high humidity or in tight fitting culture dishes .... ________

Membrane Filter Colony Counting

Fluorescent light positioned for maximum reflection of colonies with metallic yellowish surface luster .... ________
Colonies uniformly dispersed over effective filtration area .... ________
Special repeat samples requested when coliforms are "TNTC" or colonial growth is confluent, obscuring coliform detection .... ________
Total coliform count calculated in density per 100 ml .... ________

Verification of Total Coliform Colonies

When coliforms found in potable water, verified all or 10 percent to lactose or lauryl tryptose broth; then to BGLB broth .... ________
Coliform on one set of critical stream pollution samples to be used in enforcement action verified .... ________

Choice of Total Coliform Methods

M-Endo MF broth or LES Endo agar used in a single step procedure ..... ________
Incubated MF on LST absorbent pad for 1½ to 2 hours at 35°C; then on M-Endo broth or LES Endo agar for 20 to 22 hours at 35°C .... ________
Enrichment procedure evaluated for optimum recovery of stressed coliforms in chlorinated waters and industrial wastes .... ________

Fecal Coliform Membrane Filter Procedure

________________ dilution water; ________ (pore size) MF
Following filtration, MF placed over pad saturated with M-FC broth ..... ________
MF culture placed in waterproof plastic bag and submerged in water bath within 30 minutes .... ________
Incubated at 44.5°C ± 0.2°C for 24 hours .... ________
Counts made promptly after removal from the incubator .... ________
Blue colonies counted as fecal coliforms .... ________
Count calculated in density per 100 ml .... ________

CHAPTER IX
SUPPLEMENTARY BACTERIOLOGICAL METHODS

Monitoring water quality may occasionally require the use of supplementary microbiological tests that are specific for a particular need. The standard plate count is recommended for measurement of the general bacterial population in potable water. This population must be controlled to reduce the potential public health risk due to secondary pathogenic bacteria and to minimize interference with the detection of low levels of coliforms. In swimming pool water (which has not been considered in this Handbook), the standard plate count is the most important test for determining the efficacy of the disinfection process. At the same time, detection of *Staphylococcus aureus* and *Pseudomonas aeruginosa* populations present in the water provides an important index of potential skin, eye, ear, and nose infections. Although the primary bacteriological parameter for monitoring fecal contamination of naturally occurring recreational waters is the fecal coliform test, parallel examination for fecal streptococci is of value in interpreting the sources of fecal contamination, i.e., from domestic wastes or from farm animals or stormwater runoff.

Water pollution investigations may require the search for *Klebsiella pneumoniae* to demonstrate that excessive concentrations of nutrients are discharged in poorly treated paper mill or textile processing wastes. In stream, lake, and estuarine field studies, qualitative tests for *Salmonella* occurrence are often requested to demonstrate pathogen discharge in effluents or pathogen persistence in receiving waters. Serotype identification of *Escherichia coli* and the isolation of enteropathogenic *E. coli* may be of value in some epidemiological studies of waterborne outbreaks.

In areas remote from the laboratory, where samples cannot be received within the specified time limit of 30 to 48 hours for potable waters or 6 hours for water pollution samples, the use of a delayed total coliform test or fecal coliform procedure may be desirable. The entire testing procedure may be done at the field site with the use of a MF field laboratory kit or equivalent. Finally, during periods of emergency, a rapid (7-hour) fecal coliform procedure may be a desirable supplement to the standard potable water analyses for quality assessment or in monitoring natural bathing waters for data used to support decisions governing beach closures and prompt reopenings.

STANDARD PLATE COUNT

The EPA's *Primary Drinking Water Standards* (1) specify that public water supplies provide a potable water with no greater than 500 organisms per 1 ml as determined by the standard plate count. This measurement can be a valuable criterion for detecting water quality deterioration in supply

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distribution lines and storage reservoirs and for indicating the magnitude of excessive bacterial populations (populations that may even suppress visible gas production by coliforms and produce false-positive results in the multiple tube test or overgrow membrane filter cultures, masking the detection of total coliforms). The standard plate count limit would also indirectly restrict the occurrence and magnitude of ***Pseudomonas***, *Flavobacterium*, and other secondary pathogenic invaders that could pose a health risk in the hospital or in a similar environment of susceptibles.

The application of a bacterial density limit to potable waters and swimming pool samples requires strict adherence to a specific protocol that yields reproducible results and measures a standardized population. Sample bottles must be sterilized no more than 30 days before use; the purpose of this time restriction is to reduce the possibility of chance contamination in the sterile bottle supply during the storage period.

Samples collected for the standard plate count determination must be transported to the laboratory as quickly as possible and immediately processed to prevent significant bacterial density changes. These samples may be transported without refrigeration only when the elapsed time between sample collection and sample processing in the laboratory does not exceed 8 hours. This transit time may be extended to periods up to 30 hours only if the samples are transported in iced containers (2).

Before preparing pour plates, shake the sample vigorously (approximately 25 times) to ensure proper distribution of the organisms within the water sample. Some laboratories use a dilution bottle mechanical shaker for vigorous sample agitation; however, such equipment is optional. Limit the number of samples agitated on a mechanical shaker to four; this will minimize bacterial stratification due to sedimentation of turbidity particles during the time period between shaking and sample examination.

Triplicate sample portions are recommended to ensure optimum data precision although duplicate plate counts are acceptable for routine analyses. Sample portions of 1 ml and 0.1 ml may be pipetted directly into the culture dish; portions of 0.01 ml must be prepared by dilution and 1 ml of the appropriate dilution used. While pipetting sample volumes, do not immerse the pipet more than 1 inch (2.5 cm) below the surface of the sample or dilution. This will reduce the uncontrolled drainage of sample from the outside of the pipet to the pour plate in preparation. Allowing the portion of the pipet that was immersed to contact the inside of the sample container upon withdrawal will also reduce the amount of liquid adhering to the outer pipet walls. Hold the pipet at an angle of about 45 degrees with the tip touching the inside bottom of Petri dish when dispensing the sample. After the sample portion is delivered, gently touch the pipet tip once against a dry spot in the culture dish bottom and withdraw the pipet. Blow-out type pipets are not acceptable unless the mouth-end is plugged with cotton. Gently blow out residual sample portion only in those cases where such pipets are used. This protocol must be rigidly adhered to so that replicate sample portions do not produce irregular colony counts.

The estimated amount of agar needed for preparation of the pour plates should be available immediately after the portions are dispensed. No more than 20 minutes should elapse in this procedure to prevent bacterial density changes in the sample portion. Melt the required amount of agar in a boiling water bath or in flowing steam not exceeding atmospheric pressure. Before use, temper the melted agar in a water bath to 44° to 46°C. If the agar temperature exceeds these limits, some viable bacteria will be killed. To determine melted agar temperature, use a separate flask or bottle, identical to that used for sterile medium, containing glycerine (or medium) and an accurate thermometer with bulb immersed in the liquid. This blank flask or bottle should be exposed to the melting and cooling procedures along with each lot of medium used. Do not melt more agar than will be used within a 3-hour period to avoid development of insoluble phosphate in the clear medium. These particles can be confused with bacterial colonies during the counting procedure.

Flame the lip of media containers immediately before and periodically during pouring into the culture dishes. Do not add less than 10 ml of melted medium to the 100-mm-size culture dishes. The liquid agar and sample portions are then thoroughly mixed by gently rotating the Petri dish to spread the mixture evenly. It is also recommended that one control plate (no sample added) should be prepared for each bottle of agar used to verify that the agar was sterile before use. After the agar has solidified, invert the pour plates and place in the incubator. Plate inversion during incubation prevents condensation from dropping onto the agar surface.

The mandatory use of standard plate count agar and incubation of all pour plate cultures of potable and swimming pool water samples at 35° ± 0.5°C for 48 ± 3 hours is essential. Many organisms in these samples have been physiologically stressed, and this results in slow initial growth in culture media. Bottled water and stored emergency water supplies must be incubated at 35° ± 0.5°C for 72 ± 4 hours because many of the bacteria in these waters demonstrate a prolonged lag phase during adaptation to growth on tryptone glucose extract agar or plate count agar (3).

Count all colonies on selected plates promptly after the incubation period. If counting must be delayed temporarily, store plates at 5° to 10°C for a period of no more than 24 hours, but avoid this as routine practice. Record the results of sterility controls on the report form for each lot of samples.

A Quebec colony counter, preferably a Darkfield model that reduces the light glare, is used to count all colonies on each plate, including pinpoint-sized colonies. Avoid mistaking precipitated matter in the media for pinpoint colonies. When spreading colonies are encountered, count each chain of colonies originating from a separate source as one colony. Do not count each individual growth in a spreader chain as a separate colony. Each technician should be able to duplicate his count on the same plate within 5 percent; different individuals counting the same plate should be within 10 percent. Automatic plate counting instruments utilizing a television scanner and an electronic counter are now available. Such instrumentation is acceptable if parallel evaluation with manual counting gives comparable results.

After incubation, choose, from each sample, sets of replicate plates that contain between 30 and 300 colonies per plate. Compute the bacterial count per milliliter by multiplying the average number of colonies per plate by the dilution used and report as the "Standard Plate Count" per milliliter.

If there is no plate with 30 to 300 colonies, and one or more plates have more than 300 colonies, use the plate(s) having a count nearest 300 colonies. Compute the count by multiplying the average count per plate by the dilution used and report as the "Estimated Standard Plate Count" per milliliter.

If plates from all dilutions of any sample have no colonies, report the count as less than one (< 1) times the corresponding lowest dilution. For example, if no colonies develop on the 1:100 dilution, report the count as "less than 100 (< 100) Estimated Standard Plate Count" per milliliter.

If the number of colonies per plate far exceeds 300,do not report the result as "too numerous to count" (TNTC). If there are fewer than 10 colonies per cm^2, count colonies in 13 squares (of the colony counter) having representative colony distribution. If possible, select seven consecutive squares horizontally across the plate and six consecutive squares at right angles; be careful not to count a square more than once. Multiply the sum of the colonies in 13 representative square centimeters by 5 to compute the estimated colonies per plate when the area of the plate is 65 cm^2. When there are more than 10 colonies per cm^2, count four representative squares, take the average count per square centimeter, and multiply by the appropriate factor to estimate the colonies per plate (usually about 65). When bacterial counts on crowded plates are greater than 100 colonies per cm^2, report the result as greater than (>) 6,500 times the highest dilution plated.

If spreading colonies (spreaders) are encountered on the plate(s) selected, count colonies on representative portions only when (a) colonies are well distributed in spreader-free areas, and (b) the area covered by the spreader(s) does not exceed one-half the plate area.

When spreading colonies must be counted, count each unit of the following types as one: (a) the first is a chain of colonies that appears to be caused by disintegration of a bacterial clump as the agar and sample were mixed. Count each such chain as a single colony; do not count each individual colony in the chain; (b) the second type of spreader develops as a film of growth between the agar and the bottom of the Petri dish; (c) the third type forms in a film of water at the edge or over the surface of the agar. Types (b) and (c) largely develop because of an accumulation of moisture at the point from which the spreader originates. They frequently cover more than half the plate and interfere with obtaining a reliable plate count.

If plates prepared from the samples have excessive spreader growth, report as "Spreaders" (Spr). When plates are uncountable due to missed dilution, accidental dropping and contamination, or the control plates indicate that the medium or other material or labware was contaminated, report as "Laboratory Accident" (LA).

When colonies on replicate plates, or consecutive dilutions, or both, are counted and the results are averaged before recording, round off counts to two significant figures only at the time of converting the calculation to standard plate count per milliliter.

Avoid creating fictitious ideas of precision and accuracy when computing standard plate counts: record only the first two lefthand digits. Raise the second digit to the next highest number only when the third digit from the left is 5, 6, 7, 8, or 9; use zeros for each successive digit toward the right from the second digit. For example, a count of 142 is recorded as 140, and a count of 155 as 160, whereas a count of 35 is recorded as 35.

Staphylococcus

Since a specific direct method is not available for detecting *Staphylococcus aureus* in swimming pool water, partition counts must be made on media used for total *Staphylococcus* determinations (4-7). *Staphylococcus* total counts can be made using Chapman-Stone agar, M-*Staphylococcus* broth, or Vogel-Johnson agar in conjunction with the MF procedure; incubate 48 hours at 35°C. Although typical *Staphylococcus aureus* colonies may appear on Chapman-Stone agar and M-*Staphylococcus* broth cultures as yellow pigmented colonies or on Vogel-Johnson agar as shiny black colonies, individual samples may contain stressed strains that fail to pigment on the MF. Detection of *Staphylococcus aureus* in natural bathing waters may require the addition of 0.7 mM sodium azide to M-Staphylococcus agar (8) and the inclusion of lipase manitol salts agar (9) in the isolation procedure to suppress interferences from Gram negative bacilli (10). Therefore, a proportion of all colonies present on the MF culture must be verified to determine the percent occurrence of *Staphylococcus aureus*. It will be necessary to replicate from these MF cultures or verify a percentage of individual colonies as positive in both coagulase medium and dextrose broth as well as demonstrate that these catalase positive organisms are Gram positive cocci.

Pseudomonas aeruginosa

If *Pseudomonas aeruginosa* is present in potable water supplies or recreational waters, it can be detected by using M-PA agar and the MF procedure (11) or by using asparagin broth with confirmation in acetamide medium in a multiple tube procedure.

MF cultures on M-PA agar are incubated at 41.5°C for 48 hours. Colonies of *Pseudomonas aeruginosa* on this medium have a flat appearance with darkish-brown to greenish-black centers surrounded by an opaque to translucent white periphery. Colony verification involves streaking individual plates of Brown — Scott Foster milk agar (12) with selected isolated colonies from the MF culture. The milk agar plates are incubated at 35°C for 24 hours. *Pseudomonas aeruginosa* hydrolyzes the casein and produces a yellowish-green to green diffusible pigment.

In the multiple tube procedure, inoculate sample portions into asparagine broth; use single-strength broth for sample volumes of 1 ml or less and double-strength broth if 10 ml inocula are required. Incubate all tubes at 35° to 37°C. After 24 and 48 hours of incubation, examine each tube under black light for production of a greenish fluorescence in the culture. Such observations constitute a positive presumptive test. Confirm positive presumptive tubes by transferring a loop full of broth to either acetamide agar slants or acetamide broth. A positive confirmed reaction is indicated by a purple color (high pH) development within 48 hours at 35° to 37°C.

In those waters that exhibit significant false positive results in the presumptive test, change the incubation temperature to 39°C to reduce the recovery of fluorescent pseudomonads such as *Pseudomonas fluorescens* and *Pseudomonas putida* (13). To further verify positive acetamide tubes, transfer growth to A medium (King, Ward, and Raney) for a test of pyocyanin production (14).

FECAL STREPTOCOCCI

The use of the MF method or agar pour plate technique for fecal streptococci detection is recommended over the multiple tube procedure for the following reasons: (a) recoveries on MF media currently in use are higher and less affected by interference organisms; (b) greater numbers of false positive reactions occur in broth MPN systems; and (c) when group or species identification is required, MF plates and agar pour plates readily allow for primary isolations of fecal streptococcus colonies. However, the *Standard Methods* (15) multiple tube procedure that employs azide dextrose presumptive broth (16) and ethyl violet azide confirmatory broth (17) must be used on waters with high turbidities that interfere with membrane filtration.

Media available for use with the MF procedure include M-Enterococcus agar (18) and KF Streptococcus agar (19). Both media give equivalent results when domestic sewage is examined because *Streptococcus fecalis* and its biotypes are the predominant fecal streptococci in domestic wastes. However, the recovery of *Streptococcus bovis* and *Streptococcus equinus*, both of which are common to feedlot runoff and meat packing operations, is much better on KF agar. For this reason, KF agar is the recommended medium for many water pollution investigations.

Where the pour plate method is preferred, KF Streptococcus agar or PSE agar (20,21) may be used since these two media give essentially equivalent streptococcus recoveries. However, PSE pour plates require only a 24-hour incubation period whereas KF agar must be incubated 48 hours to permit optimum fecal streptococcus colony development. When chlorinated sewage effluent and water samples with high turbidity must be examined, use the pour plate technique, with either PSE or KF agar, in preference to the MF procedure.

Normally there is no need for species identification of fecal streptococci in stream pollution studies. Density relationships with fecal coliforms are adequate to assign the probable source of waste discharge as being domestic or from farm animals and wild life. However, special applications involving tracer organism identification, confirmation of sanitary significance of very low fecal streptococcus densities, and media evaluations will require further biochemical identification. The basic biochemical tests (Figure 6) include observation of growth in brain-heart infusion broth within 2 days at 45°C, and within 5 days at 10°C; plus confirmation for growth at 45°C in 40 percent bile and a negative catalase reaction. Beyond this point, further choice of biochemical tests varies in number and kind depending upon the researcher's viewpoint (22-26). Practical application of identification procedures demands a simplification of the tests and more specific biochemical reactions. Further de-

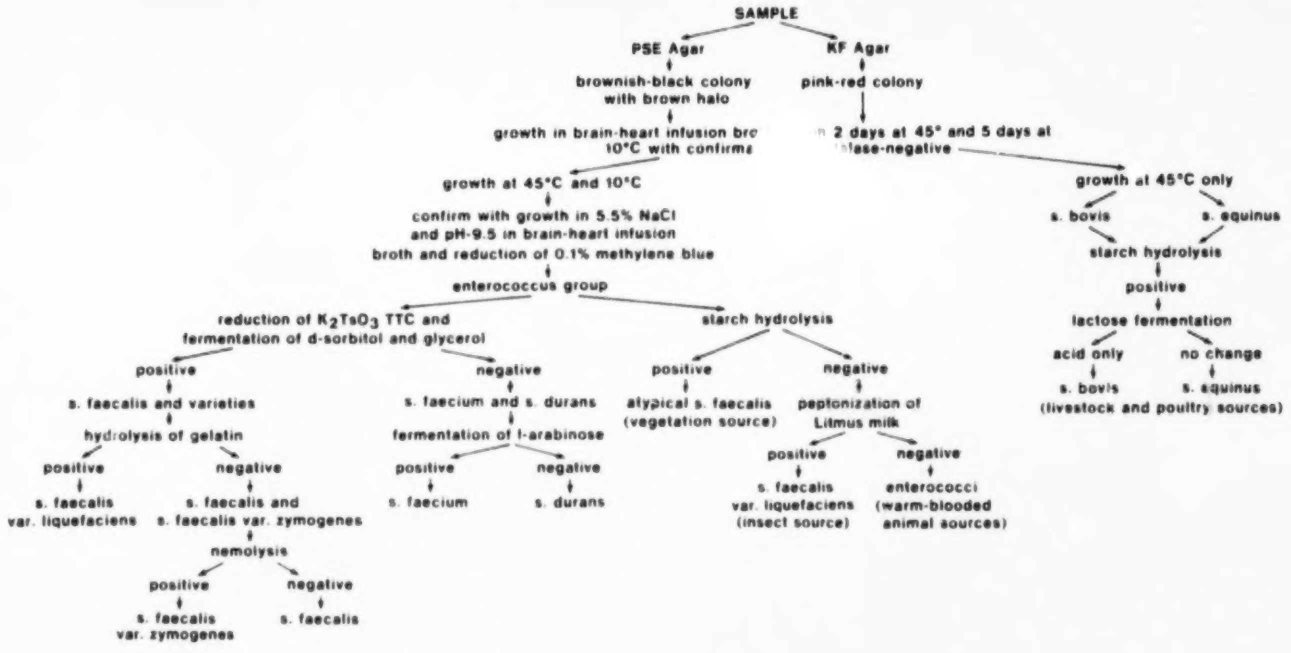
SAMPLE
PSE Agar
KF Agar
brownish-black colony with brown halo
pink-red colony
growth in brain-heart infusion bro
10°C with confirme
n 2 days at 45° and 5 days at
talase-negative
growth at 45°C and 10°C
confirm with growth in 5.5% NaCl and pH-9.5 in brain-heart infusion broth and reduction of 0.1% methylene blue
enterococcus group
reduction of K2TeO3 TTC and fermentation of d-sorbitol and glycerol
positive
s. faecalis and varieties
hydrolysis of gelatin
positive
s. faecalis var. liquefaciens
negative
s. faecalis and s. faecalis var. zymogenes
hemolysis
positive
s. faecalis var. zymogenes
negative
s. faecalis
negative
s. faecium and s. durans
fermentation of l-arabinose
positive
s. faecium
negative
s. durans
starch hydrolysis
positive
atypical s. faecalis (vegetation source)
negative
peptonization of Litmus milk
positive
s. faecalis var. liquefaciens (insect source)
negative
enterococci (warm-blooded animal sources)
growth at 45°C only
s. bovis
s. equinus
starch hydrolysis
positive
lactose fermentation
acid only
s. bovis
no change
s. equinus
(livestock and poultry sources)

Figure 6. Schematic Outline for Identification of Fecal Streptococci

velopment of a serological schema, which currently includes 39 serotypes, could be an important breakthrough in this problem (25,27-32).

Klebsiella

Specific differential media for the detection of *Klebsiella* are being investigated. The use of a nitrogen-deficient medium (33) is a promising approach for differentiating between *Klebsiella* and *Enterobacter*. On this medium, *Klebsiella* colonies are larger in size and more convex and mucoid in appearance than are *Enterobacter*. Since *Klebsiella* are coliforms, the use of M-Endo MF and the MF procedure may be the most practical means for primary isolation since this medium is commonly available in most laboratories. All typical coliform colonies or a significant percentage of these colonies are then purified and submitted to the oxidase test, lactose fermentation, and the HOMoC series (hydrogen sulfite, ornithine decarboxylase, motility, and citrate utilization) of biochemical tests. *Klebsiella* biochemical characteristics are: oxidase negative, lactose positive, hydrogen sulfite negative, ornithine decarboxylase negative, motility negative, and utilize citrate as the sole source of carbon (34-36). The IMViC biochemical reactions (indole, methyl red, Voges-Proskauer, and citrate) for *Klebsiella* are − − + +, identical to *Enterobacter (Aerobacter) aerogenes*.

Salmonella

The most logical approach to *Salmonella* quantitation would be applying the MF procedure since this method has the advantage of large volume analysis, limited only by the turbidity of the sample. Unfortunately, there is only one quantitative MF method available for *Salmonella* detection, M-Bismuth Sulfite broth (37), and it is essentially specific for *Salmonella typhosa* detection, with poor recovery for most of the other 1200 *Salmonella* serotypes that might be encountered. Other quantitative methods have been proposed, but they also use the multiple tube principle and involve complex manipulations that lack the selectivity necessary for use with water samples. Apparently the excessive bacterial flora in grossly polluted waters overwhelms the selective, suppressive action of media currently in use for *Salmonella* recovery.

For these reasons, emphasis has been placed on qualitative methods for *Salmonella* detection with the further understanding that there is no single optimum concentration method, enrichment procedure, selective differential medium, or incubation temperature that will ensure the recovery of all *Salmonella* strains present in polluted water. Thus, several alternative choices in methodology must be considered, and the final decision depends upon the type of water to be examined.

There are three basic concentration techniques for *Salmonella* recovery from water: (a) sterile gauze pads submerged 3 to 5 days in water at a sampling site, with entrapped water expressed from pad to enrichment media (Moore pad technique); (b) filtering 2-liter sample volumes through diatomaceous earth held in place in the MF funnel by an absorbent pad followed by the addition of portions of the plug to enrichment media; and (c) filtration of a large volume sample through the MF, which in turn is added to a suitable enrichment broth. Gauze pads have been very useful in recovering *Salmonella* from natural waters that are free of excessive debris. The diatomaceous earth procedure will often produce better results where floating solids are present, such as in sugar beet effluents and paper mill wastes and most estuarine environments. MF filtration is often more useful when investigating contaminated wells or suspect potable water supplies. *Salmonella* have also been successfully isolated from a potable water supply by selecting M-Endo MF cultures that contain significant background growth and total coliforms, after counting, and placing the entire MF with the mixed growth into 10 ml of tetrathionate broth (containing 1:50,000 brilliant green dye) for *Salmonella* enrichment (38). This unique approach requires no special large sample collections and can be an extension of the routine total coliform analysis. For large volume samples, the transit time must not exceed 6 hours, and the initial processing, once received in the laboratory, must begin promptly.

Selective enrichment for *Salmonella* recovery from gauze pads, diatomaceous earth, or the MF into dulcitol selenite broth, tetrathionate broth, or GN broth is necessary to enrich the growth of *Salmonella* while suppressing the coliform population from a sample. Extending the incubation time from 1 to 5 days with daily streaking of cultures enhances the recovery of all *Salmonella* serotypes that might be present. Further separation of *Salmonella* strains from other members of the bacterial flora in feces and polluted fresh water has been accomplished by using a variety of enrichment-plating — incubation-temperature combinations: 37° to 37°C; 37° to 41.5°C; and 41.5° to 37°C (39-41). The recovery of *Salmonella* organisms from solid waste — sludge mixtures, municipal solid waste, and incinerator residue and quench water has been found to be optimal when enrichment incubation at 39.5°C for 16 to 18 hours is used (42). *Salmonella* detection in estuarine waters (using the same enrichment media), however, appears to be more successful when 37°C is the chosen incubation temperature (43). These observations demonstrate that the choice of media, incubation temperature, incubation time, and water sample source are interrelated factors that influence *Salmonella* recovery. Therefore, a variety of plating media should be used to isolate *Salmonella* strains from enrichment cultures. Brilliant green agar, Hektoen enteric agar, xylose lysine desoxycholate agar, and bismuth sulfate are most often chosen because of their more selective recovery.

Preliminary *Salmonella* Screening

From each selective medium, choose isolated colonies of *Salmonella*-like appearance. Restreak these strains on the differential media to obtain pure cultures before proceeding to the preliminary screening procedures. If the laboratory has fluorescent antibody (FA) capability, it is recommended that suspect cultures be given a preliminary screening in this technique before proceeding to a study of differential test reactions. Since the FA technique does have cross-reaction problems that produce false positives, this method can only be recommended as a rapid gross screening of suspect colonies to eliminate a variety of strains that would otherwise require submission to selected differential tests.

In the FA technique (44), a light saline suspension is prepared from an 18- to 24-hour agar slant pure culture. Smears of this suspension are then prepared on clear-glass FA slides (1.0 to 1.1 mm thick). After the smears are air dried, they are fixed for 2 minutes in Kirkpatrick's fixative, rinsed briefly in 95 percent ethanol, and allowed to air dry. Do not blot dry these preparations. Once the fixed smears are dry, cover with one drop of *Salmonella* polyvalent OH conjugate, previously diluted 1:8. Place the slides in a moist chamber to prevent evaporation of the staining reagent, and after 30 minutes, wash away excess reagent by dipping each slide in buffered saline (pH 7.5 to 8.0). Then place each slide in a second bath of buffered saline for 10 minutes, remove, rinse in distilled water, and drain dry. Place a small drop of mounting fluid on the smear and cover with a No. 1 coverslip. Examine under fluorescence scope, using UG-1 (2 mm) primary filter and GG-9 (1 mm) ocular filter for evidence of a positive agglutination fluorescence.

In those laboratories without FA test capabilities, suspect colonies are further characterized by a study of fermentation reactions in triple sugar iron agar and reactions to indole, motility, urease, and lysine decarboxylase. Commercial differential media kits are also available for use in this preliminary screening procedure before serological confirmation (45,46). Although test reactions from these kits may range from 95 to 98 percent agreement with conventional tests, some significant individual differences may occasionally occur. In some instances, supplemental tests will be necessary to further differentiate among strains of the large group of *Enterobacteriaceae*.

Serological Grouping of *Salmonella*

Upon completion of the recommended biochemical tests used to tentatively identify suspected colonies as *Salmonella*, inoculate the pure culture strain (if necessary, re-streak culture on one of the differential agars to check for purity) into trypticase soy broth or brain heart infusion broth. Then incubate the inoculated broth for 24 hours at 37°C. To ensure maximum culture vigor, transfer the strain through several fresh tubes of brain heart infusion broth before a final inoculation onto slants of brain heart infusion agar. The culture is then ready for the slide agglutination test.

Subdivide a glass slide into appropriate squares, using a thick wax pencil (47). Place a drop of *Salmonella* "O" polyvalent antiserum on one square, antiserum plus 0.85 percent sodium chloride on a second square, bacterial suspension in 0.85 percent sodium chloride on another square, and bacterial suspension in 0.85 percent sodium chloride plus antiserum on a fourth square. Gently rock the slide for a maximum of 2 minutes and observe for development of an agglutination reaction in the fourth square only. If agglutination occurs, repeat the slide agglutination procedure using the specific *Salmonella* "O" antiserum groups for serotype identity. For those cultures that do not react to specific "O" *Salmonella* antiserums, repeat the slide agglutination procedure using *Salmonella* Vi antiserum. A negative agglutination response in *Salmonella* Vi antiserum indicates the strain is not of the *Salmonella* genus and can be discarded. If agglutination does occur, then heat the culture in a boiling water bath for 10 minutes, cool, and retest with the individual *Salmonella* "O" antiserum groups and *Salmonella* Vi antiserum. As a recommended procedure, submit the tentatively identified culture to a certified public health laboratory or national typing center for verification of serotype.

Salmonella isolations from water samples should only be conducted in laboratories that have personnel experienced in medical bacteriology or trained in these procedures. The search for pathogens in water requires certain expertise in selecting methodology and media and in interpreting results. Such personnel may not be available in the small laboratory limited to routine water testing.

PATHOGENIC LEPTOSPIRES

The presence of pathogenic leptospires in natural waters is extremely variable and is complicated by many factors that make interpretation of results extremely difficult. These include intermittent leptospire discharge from infected wildlife or farm animals near the watercourse, stormwater runoff, and flooding of contaminated land areas in the watershed (48). Favorable persistence in warm, slow-moving waters having a pH of 6.0 to 8.0 (49-52) and moderate levels of bacterial nutrients (53) also complicates the interpretation of leptospire occurrences. Even when pathogenic leptospires are present, their detection is difficult because of the competitive growth of other organisms (54) and the necessity to differentiate between pathogenic and saprophytic strains (50,54-57). Keep in mind, therefore, that failure to isolate pathogenic leptospires from natural waters does not necessarily indicate their absence.

These factors explain why qualitative methodology has evolved to concentrate leptospires from water. Long-term incubation on various media is necessary because of the relatively slow growth of the organisms in the laboratory. During incubation, inoculated media are repeatedly checked for the appearance of leptospires and for culture contamination. Upon detection, various biochemical responses supplemented by serological identification can be used to separate pathogenic and saprophytic strains of leptospire isolates. Animal tests for pathogenic leptospires are also recommended, but these should be done on primary pure culture isolates since pathogenic strains may become avirulent through subsequent culture passages.

Preliminary Concentration

Pathogenic leptospira may be concentrated in near-shore bottom sediments of streams and farm ponds. Therefore, gentle agitation of bottom sediment before sampling is recommended to ensure collection of bacteria-laden material from the sediment-water interface. The bacteriological bottom sampler may also be used to collect this finely suspended material in sterile plastic bags. Upon return to the laboratory (or, preferably, to a field site), vigorously shake the sample to release entrapped bacteria from the sediment and immediately prefilter through either a Whatman No. 1 filter paper or a MF absorbent pad to remove heavy turbidity. Pass the prefiltered sample through a Swinney hypodermic adapter containing a fiber glass prefilter and a MF of 0.45-micron pore size to separate leptospires (which can pass through the pores into the filtrate) from other organisms present in the sample (that are retained by the MF).

Cultivation

Portions of sample filtrate (1-ml and 0.1-ml quantities) may then be inoculated into Fletchers semisolid medium containing 10 percent rabbit serum (58). Incubate the inoculated medium at 30°C for 6 weeks, and examine each tube (using darkfield illumination and 250 × magnification) at least once a week for leptospiral growth and culture contamination (59). Strains of *Vibrio* or *Spirillum* are the most common contaminants observed, particularly when filtrate volumes greater than 0.1 ml are examined (58).

Leptospires are helicoidal, usually 6 to 20 microns in length, and each coil is about 0.2 to 0.3 micron in diameter. The coils of leptospires are more compact than those of other spirochaetes (59). If leptospires are not observed microscopically within a 6-week incubation period, the test is considered negative.

As an alternate enrichment procedure, spread plates of plate count agar (60) or bovine albumin polysorbate 80 medium (61,62) may be inoculated with 0.1- to 1.0-ml volumes of sample filtrate and incubated at 30°C for 7 to 9 days. When bovine albumin polysorbate 80 medium is used, an agar overlay of 0.7 percent distilled water agar is recommended. Regardless of the choice of agar medium, prepare the agar before inoculation (1 or 2 days) to condition it and, thus, promote even spreading of the inoculum over the agar surface. Use darkfield microscopy to identify morphologically all colonies that develop before conducting biochemical and serological tests or animal inoculations.

Differentiation of Leptospires

The detection of pathogenic leptospires in lakes and streams indicates leptospirosis in domestic animals and wildlife that frequent these waters, and signals the health risk to bathers using these waters. Therefore, the ability to differentiate pathogenic from saprophytic leptospire strains isolated from the water environment is of critical importance.

Culture Reactions

Saprophytic leptospires are strongly resistant to 10 micrograms copper sulfate per ml (56) or 100 microgram 8-azaquanine per ml (55) in Stuart's medium containing 10 percent rabbit serum. Only the saprophytic leptospires grow in a 10 percent rabbit serum medium at 13°C (50). In addition, saprophytic strains demonstrate higher oxidase response (63) and higher egg yolk decomposition activity (64) than pathogenic leptospires. Optimum laboratory cultivation temperature for the pathogenic leptospires is 30°C; incubate all test cultures for 5 days for organisms to reach their optimum growth phase. No single test should be used to differentiate saprophytic from pathogenic leptospires (65).

Verification of Pathogenicity

Commercial antisera are available that permit tentative identification of pathogenic leptospires. Final verification of the suspect pathogenic strain by animal testing should be conducted, but only by laboratories with established expertise in these procedures.

ENTEROPATHOGENIC *Escherichia coli*

The examination of suspect potable water supplies for enteropathogenic *E. coli* can be initiated by use of the fecal coliform MF procedure, utilizing M-FC broth (66). M-Endo broth may be used, but more effort will be needed to isolate the *E. coli* from the total coliform colonies (67). Blue colonies from the M-FC cultures are streaked and purified and IMViC biochemical reactions determined. Those strains producing gas by lactose fermentation and having a + + − − IMViC reaction are then tested serologically (68).

Three classes of antigens are important in the determination of the *E. coli* serogroups. The heat-stable and major grouping-factor "O" antigen is associated with the bacterial cell, the "K" antigen is associated with the envelope or capsule, and the "H" antigen with the flagella. The "K" antigen has three varieties (L, A, and B), which differ in heat lability and heat inactivation of binding power. Slide agglutination is employed for "O" and "K" antigen determinations, and the macroscopic tube test is recommended for confirmation of "O" antigens.

DELAYED INCUBATION COLIFORM PROCEDURES

Occasionally, it is desirable to filter samples in the field and then transport the MF cultures to the laboratory for subsequent incubation and examination. For total coliforms, use a modified M-Endo MF broth or LES Endo agar to slow bacterial growth during 1- to 3-day shipment to the laboratory for final processing (69-72). To prepare the holding medium used in the field, add 0.384 grams of sodium benzoate (USP Grade) or 3.2 ml of a 12 percent (W/V) sodium benzoate solution to 100 ml of either M-Endo MF broth or LES Endo agar. Where overgrowth from fungus colonies causes problems, adding 50 mg cycloheximide (actidione) per 100 ml of Endo holding medium is desirable.

Upon arrival in the laboratory, transfer the MF cultures to a fresh culture dish containing standard M-Endo MF broth or LES Endo agar, and incubate the plates at 35°C for 20 to 22 hours. If growth is visible at time of transfer, hold the cultures in a refrigerator until the end of the work day and incubate them at 35°C overnight (16- to 18-hour period). Then count the sheen colonies and calculate the total coliform count per 100 ml.

It is essential that the laboratory establish the validity of the delayed incubation test for total coliforms on those waters that are to be examined routinely by this procedure. Wide variations in ambient temperature and storage periods up to 72 hours before final processing of the MF cultures in the laboratory may stimulate the growth of some false positive, noncoliform organisms that are capable of partial breakdown of lactose. Once the magnitude of these occurrences has been determined, through sheen colony verification, data from the delayed incubation test for total coliform detection may be more accurately interpreted.

The delayed incubation concept can also be applied to fecal coliform measurements (73). After the water sample has been filtered in the field, place the MF in contact with an absorbent pad saturated with VFC (vitamin-free casitone) holding medium and send, via mail service, to the laboratory for final processing. Although the delayed incubation procedure will hold for up to 72 hours, the holding period should be minimal, and for some distant locations, air mail and/or special delivery should be used. Upon receipt of the samples in the laboratory, transfer the MF cultures to fresh culture dishes containing absorbent pads saturated with M-FC broth, place in waterproof plastic bags, and incubate, submerged, in a 44.5°C water bath for 22 ± 2 hours. Then count the blue-colored colonies (fecal coliforms) and calculate the count per 100 ml.

For verification, inoculate selected colonies into lactose broth or lauryl tryptose broth for incubation at 35°C. Transfer inoculum from each culture showing gas production in 24 or 48 hours to individual EC broth tubes for verification of lactose fermentation at 44.5°C in 24 hours. The verification procedure is recommended as an initial control check of the delayed procedure when the delayed incubation procedure is to be used for the examination of source waters on a continual basis. This check on the validity of delayed test results for specific waters may also be useful to demonstrate the test accuracy when laboratory data are to be used in enforcement actions.

RAPID METHODS

Rapid assessment of the sanitary quality of water is often needed for emergency or temporary potable water supplies, bathing beaches whose quality may have deteriorated following storms, and shellfish growing areas subject to sewage pollution. One approach to the quick determination of water quality has utilized C^{14}-labeled sodium formate in a rapid (4-hour) test for total coliforms (74,75). The procedure shows considerable promise when used for fecal coliform detection but must be refined for greater reproducibility and increased sensitivity to coliform concentrations below 100 organisms per 100 ml. A membrane filter — fluorescent antibody (MF-FA) technique has also been proposed for the rapid identification of fecal coliforms (76,77). Before the MF-FA test for fecal coliforms can be considered practical, however, commercial polyvalent antisera must be developed that include all 145 "O" antigens and 86 "K(B)" antigens identified with the *E. coli* group. In addition, antigens for a few *Enterobacter* and *Klebsiella* strains, which are also defined as fecal coliforms, must be included. At present, the three commercially available polyvalent antisera contain only 20 "O" and "B" serotypes.

Such rapid methods as these may not be adaptable to true emergency situations where skilled personnel and specialized equipment are not available. At present, the most promising approach involves the use of a new MF procedure utilizing a lightly buffered, lactose-mannitol-based medium (M–7-hour) containing an acid-sensitive indicator system. Following filtration, cultures in this procedure are incubated submerged (using waterproof plastic bags) in a 41.5°C water bath for 7 hours (78,79). Colonies must be examined at 10 to 15 × magnification using either a fluorescent light or an incandescent microscope light with a blue filter. Fecal coliform colonies appear yellow, generally very bright and distinct, but all colonies having a yellow appearance should be counted.

Results from a study of fecal coliform differentiation showed that 94.3 percent of 4,082 yellow colonies from the 7-hour medium verified as fecal coliforms and, from the same samples, 93.7 percent of 4,034 blue colonies on the M-FC control medium, after a 24-hour incubation, verified as fecal coliforms. These data indicate that both media measured essentially the same population of bacteria.

To verify fecal coliform colonies on the M–7-hour medium, transfer inocula from yellow colonies into individual tubes of lactose broth or lauryl tryptose broth for gas production at 35°C within 48 hours. Then inoculate growth from each positive lactose tube into EC broth for confirmation of gas production at 44.5°C for 24 hours. Initial verification of the colonies on the 7-hour medium is desirable to demonstrate the effectiveness of the medium to the technician using the procedure for the first time.

During periods of emergency water testing, it is suggested that the rapid test be used to supplement the standard MF total coliform test. This protocol will permit the rapid detection of gross fecal contamination in 7 hours while awaiting the 24-hour test results for a total coliform limit of one total coliform per 100 ml.

PORTABLE FIELD LABORATORY PROCEDURES

By virtue of both the simplicity of operation and the compactness of essential apparatus, the MF procedure readily lends itself to field applications. These investigations may involve routine water quality monitoring in remote areas or be of a preliminary survey nature before initiating an in-depth field study. Recognizing the potential of this procedure for monitoring potability of water supplies used in military operations, Col. Thomas Sparks and his laboratory staff at Fort Sam Houston designed and field-tested a MF portable laboratory package that had the general configuration of a suitcase the size of a picnic cooler. Within the fiber glass carrying case was the filtration funnel, plastic Petri dishes, ampouled or preweighed media vials, a hand-pumped vacuum source, a suitable electrical incubator designed to operate on 6, 12, and 24 volts DC or 115 and 230 volts AC or DC, plus other small items associated with the technique. With such a kit (Millipore Portable Water Laboratory, XX63–001–50), the properly trained technician can test approximately 24 water samples, as prescribed in *Standard Methods* (11), for total coliform analysis. A portable heat sink block (Millipore XX63–004–00 or equivalent) is an available accessory that can be used for field incubation of MF cultures for fecal coliform determinations.

Several different methods of medium preparation can be used in conjunction with the portable field laboratory—ampouled M-Endo medium is the most convenient. Each ampoule contains sufficient sterile, prepared medium to saturate one absorbent culture pad. Shelf life for ampouled M-Endo medium is approximately 18 months. Supplies must be stored at refrigerated temperatures (4° to 10°C) during this period and protected from excessive light exposure. Ampoules that appear turbid or dark red in color may be contaminated and should not be used. Another approach to media supplies for field use is to prepare, or purchase, vials of preweighed dehydrated M-Endo medium. When needed, the desired number of preweighed vials of medium are reconstituted with the appropriate amounts of distilled water, 2 percent ethanol (not denatured) is added, and the medium is carefully heated to dissolve the ingredients. The finished medium preparation is then dispensed in 2-ml volumes into culture dishes containing absorbent culture pads. Poured agar plates of the appropriate medium previously prepared in the laboratory may also be used in the field kit. Dehydrated medium pads that are reconstituted by adding 2 ml of sterile distilled water are not recommended, however, because the shelf life of this version of M-Endo medium is approximately 3 months or less and the medium quality is not uniform.

Other available field testing procedures that utilize the MF for bacterial cultivation include a multi-purpose disposable filtration culture device (the field monitor). This device serves initially as the filtration chamber and then as the culture package when a modified Endo formulation is injected into a pad below the filter. The unit is then ready for incubation and subsequent colony counting. Several independent evaluations of the field monitor concept for total coliform recovery from polluted water indicate that only 70 percent recovery of the known bacterial density is being obtained. The remaining organism loss occurs from: (a) some bacterial bypass around the filtration area to the pad below the membrane or directly to discharge through the bottom port and (b) failure of some debilitated or stressed cells to grow on the membrane and medium. In an attempt to seal off the bypass loss, the manufacturer has added a hydrophobic substance to the outer periphery of the filter. Inclusion of a consistent amount of normal-strength medium is dependent on displacement of the water entrapped in the pad with 1.3 times normal-strength ampouled medium filtered through the field monitor following water sample filtration. Vapor blockage and uneven flow-through will result in uncertain medium concentrations in the pad substrate and will ultimately affect bacterial growth. Ampouled media have a limited shelf life that must be recognized by the laboratory—6 months for M-FC and 18 months for M-Endo when stored in the dark, preferably at refrigeration temperatures.

A bacteriological "dip-stick" has also been developed that appears to offer the ultimate yet achieved in test simplicity at some sacrifice in sensitivity and flexibility. This device consists of a sterile, rectangular-shaped MF positioned above a medium-impregnated pad, both of which are secured to a plastic frame that is inserted into a mating plastic case. The basic principle of operation is the controlled absorption of 1 ml of sample through the membrane to the medium-impregnated pad of critical thickness when the dip stick is held in a water sample for approximately 30 seconds. The small volume of sample makes the dip stick self-limiting for total coliform analyses in potable water because the test baseline is established as less than one coliform per 100 ml. Preliminary evaluation of the dip stick for use as a standard plate count measurement in potable water, when compared with the *Standard Methods* procedure, shows the method to result in significantly lower bacterial counts, possibly because of the toxicity inherent in the gray-black MF and the inadequately enriched medium. The fecal coliform dip stick appears to offer the field investigator a convenient preliminary screening tool for water pollution surveys, but may yield data as much as 10-fold lower than that obtained by the fecal coliform multiple tube procedure.

These modifications of the MF test (field monitor and dip-stick) are not acceptable as a substitute for the *Standard Methods* procedures because of inadequate sensitivity for recovering 85 percent or more of the coliform population or because the test inability to measure coliforms at levels below 1000 per 100 ml of sample (10 per ml). Such tests may be useful in obtaining a quick preliminary estimate of water quality, but where the data are to be used for an enforcement action or submitted as evidence in a court of law, *Standard Methods* MF procedures or other proven methods acceptable to court must be employed.

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GUIDELINES ON SUPPLEMENTARY BACTERIOLOGICAL METHODS

Standard Plate Count

Sample bottles sterilized within 30 days .... ________
Sample transit time limited to 8 hours without cooling, or 30 hours if iced; sample shipping containers used .... ________
Sample shaken vigorously at moment of plating .... ________
Sample portions plated in triplicate .... ________
Not more than 1 nor less than 0.1 ml plated (sample or dilution) .... ________
Ten milliliters or more liquefied agar medium added at a temperature between 44° and 46°C .... ________
Melted medium stored for not more than 3 hours before use .... ________
Plates for potable waters and swimming pools incubated for 48 hours at 35°C ________
Plates for bottled waters incubated for 72 hours at 35°C .... ________
Only plates with between 30 and 300 colonies counted, except 1-ml sample with less than 30 colonies .... ________
Only two significant figures recorded and calculated as standard plate count per 1 ml .... ________

Staphylococcus

Total *Staphylococcus* count determined on (specify type of agar) ________________
Staphylococcus aureus density determined by coagulase test .... ________

Pseudomonas aeruginosa

MF cultures incubated on M-PA at 41.5°C for 48 hours .... ________
Colonies verified by Brown — Scott Foster milk agar streak plates .... ________
MPN procedure employed using asparagine broth in the presumptive test . ________
Greenish fluorescence confirmed in acetamide medium .... ________

Fecal Streptococci

Choice of procedures: multiple tube ________; MF ________; pour plate ________
Media choice ________________________________
Biochemical reactions: growth at 45° and 10°C, 40 percent bile, catalase reaction, and starch hydrolysis .... ________

Klebsiella

Primary isolations as coliforms on Endo-type medium .... ________
Biochemical tests: HOMoC series; lactose; oxidase .... ________

Salmonella

Qualitative procedures:
  Concentration method ________________________________
  Enrichment medium ________________________________
  Enrichment incubation time ________________; temperature ________________
  Choice of plating media ________________________________
Quantitative procedure:
  Salmonella typhosa quantitated using MF procedure and M-bismuth sulfite broth .... ________

Preliminary *Salmonella* Screening

FA technique:
  Light saline suspension of a 18- to 24-hour pure culture, then air dried ________
  Kirkpatrick's fixative used; rinsed in 95 percent ethanol .... ________
  Salmonella polyvalent OH conjugate, diluted 1:8 .... ________

After 30 minutes in moist chamber, excess reagent washed off in buffered saline .... ________
Washed in a second bath of buffered saline for 10 minutes .... ________
Placed small drop of mounting fluid on smear and covered with No. 1 coverslip .... ________
Examined under fluorescence scope using UG-1 primary filter and GG-9 ocular filter .... ________
Biochemical reactions included triple sugar iron agar, indole, motility, urease, lysine decarboxylase, plus others to differentiate strains encountered .... ________

Serological Grouping of *Salmonella*

An 18- to 24-hour pure culture, grown in brain heart infusion, used .... ________
Glass slide subdivided into four squares using a thick wax pencil .... ________
Square #1 contained *Salmonella* "O" polyvalent antiserum .... ________
Square #2 contained antiserum plus 0.85 percent sodium chloride .... ________
Square #3 contained bacterial suspension in saline .... ________
Square #4 contained bacterial suspension in saline plus antiserum (test square) .... ________
Checked within 3 minutes for positive agglutination in all squares .... ________
If agglutination occurred in Square #4 only (test square), procedure repeated using specific antisera for serotype identity .... ________

Pathogenic Leptospires

Preliminary concentration:
Water samples collected from near-shore bottom sediments .... ________
Turbid sample shaken vigorously, then prefiltered through paper filter ________
Prefiltered sample then passed through 0.45-μm MF and filtrate tested for leptospire occurrences .... ________
Cultivation:
Medium ________________ incubation time at 30°C ____________________
Growth verified by darkfield microscopy .... ________
Differentiated from saprophytic leptospires .... ________
Pathogenic strains serologically identified .... ________
Pathogenicity verified by animal testing .... ________

Enteropathogenic *Escherichia coli*

Primary isolations made on M-FC at 44.5°C or Endo-type medium at 35°C ________
Colonies purified and identified by biochemical procedures .... ________
Serotypes determined by slide agglutination reactions .... ________
Serotypes confirmed by the macroscopic tube test for "0" antigen reaction ________

Delayed Incubation Procedures

Total Coliforms:
After filtration, MF placed over pad of M-Endo containing 3.2 ml of 12 percent sodium benzoate solution per 100 ml of medium .... ________
Fifty milligrams of cycloheximide added per 100 ml of holding medium for fungus suppression .... ________
Culture transported by mail service to laboratory within 72 hours .... ________
MF cultures transferred to standard M-Endo medium at laboratory .... ________
Incubated at 35°C ± 0.5°C for 20 to 22 hours .... ________
If growth visible at time of transfer, held in refrigerator until end of work day then incubated at 35°C overnight (16- to 18-hour period) .... ________
Sheen colonies counted, verified if necessary, and total coliform density per 100 ml calculated .... ________

Fecal Coliforms:

- After filtration, MF placed over pad of VFC broth .... ________
- Cultures transported by mail service to laboratory within 72 hours ... ________
- MF cultures transferred to M-FC broth at laboratory .... ________
- Cultures placed in waterproof plastic bags and incubated in 44.5°C water bath for 22 ± 2 hours .... ________
- Blue colonies counted, verified if necessary, and fecal coliform density per 100 ml calculated .... ________

Rapid Methods

M-7-hour broth and MF procedure used; incubated at 41.5°C for fecal coliform detection .... ________

All yellow colonies counted, verified if necessary, and fecal coliform density per 100 ml calculated .... ________

Portable Field Laboratory

Standard laboratory MF procedures adapted to field application .... ________

Ampouled M-Endo shelf life limited to 18 months .... ________

Ampouled M-FC shelf life limited to 6 months .... ________

CHAPTER X
LABORATORY MANAGEMENT

Laboratory involvement in developing the support data used for monitoring water quality has paralleled the increased concern over environmental pollution. Bacteriological services offered by the water laboratory, in addition to the traditional examination of potable waters, may also include gathering stream pollution data, monitoring fresh and saline recreational water qualities, checking the quality of shellfish growing waters, and evaluating effluent qualities from a variety of water users. The extent of these examinations will be governed by staff size, experience and training, and laboratory space and the availability of specialized equipment and safety provisions for handling waterborne pathogen investigations.

LABORATORY RECORDS

State health and environmental laboratories and municipal water plant laboratories examine approximately 3.5 million samples annually from this Nation's public and private water supplies. Frequency of unsatisfactory samples reported from public supplies, serving some 180 million individuals, varies from state to state but most often ranges from 3 to 5 percent. By contrast, about 40 to 60 percent of all private domestic water supplies, serving approximately 33 million consumers, fail to meet the Federal Drinking Water Standards. Available national data indicate that the MF procedure is being used by 72 state and branch laboratories and by over 125 municipal laboratories. MF applications range from use on only stream pollution samples to the analysis of all public and private potable waters.

Inspection of laboratory records on the bacteriological examination of public water supplies occasionally uncovers evidence of insufficient data retention, filing backlogs, and poor data retrieval. Compilations of data on water samples examined during the year should include a breakdown on the total number of samples for each of the following waters: public, private, swimming pool, natural bathing, and stream. Records from some laboratories using the MPN test must be divided by 5 because total examinations have been padded by counting five tubes per test as five examinations.

A study of the available engineering division's records for municipal supplies may indicate that only a minimum of the information available from the laboratory water sample report is being retained. Thus, inspection of laboratory water sample reports are, in general, more meaningful in evaluating the scope of the surveillance program. In one engineering record system, only the total number of positive tubes and the total number of presumptive tubes inoculated per month were recorded. This

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made it impossible to reconstruct the MPN value for any given unsatisfactory sample or samples during the month, and the attempt to analyze these records for evidence of a repeat sampling requirement based on specific instances of unsatisfactory sample results was inconclusive.

Data submitted on water-stained report sheets must not be arbitrarily selected for use in monthly reports on public water supply monitoring only when results are satisfactory and be rejected when the laboratory findings are unsatisfactory. Any hypothesis that water-stained reports means the samples did leak in transit and become contaminated is difficult to substantiate by facts. The more logical explanation for water-stained reports is found in the common occurrence of wetness on the outside of the bottle acquired during sample collection. These water droplets then stain the laboratory report form, which often is wrapped around the bottle, then inserted into the mailing tube or sample case. Upon arrival in the laboratory, any received samples found to leak either from improper screw cap closure, defective cap liners, or cracked bottles should be rejected and the report marked with an explanation for rejection. Another sample must be immediately requested for analysis. Any further rejection of some laboratory reports based on water-stained sample sheets should be discontinued as purely speculative.

LABORATORY REPORTS

Reports on the examination of potable water samples may be prepared by the laboratory division personnel or, exclusively, by the division of engineering clerical staff. Uniformity in state record systems is rare. Report forms vary in complexity from a minimum of information on the specific sample to a detailed sanitary evaluation of the supply. The bacteriological water-sample report form for potable water must include information that identifies sample location, time and date of collection, sample collector's initials, time of receipt in the laboratory, and total coliform occurrence per 100 ml. Additional essential information spaces should be available for reporting chlorine residual, turbidity, standard plate count (48 hours at 35°C) per 1 ml, and a check box that states the sample does or does not conform to the Federal Drinking Water Standards. Finally, the form should also include a check spot to specify if the water sample is routine (part of the normal monitoring program), a recheck sample (repeat sample requested when potable water results are unsatisfactory), or a special sample. This latter information would be of assistance to the laboratory in processing samples and to the engineering section in separating repeat sample information from routine sampling data. The form sizes vary from quarter-page, half-page, and full-page, to cards used in IBM systems. Copies may be prepared with carbon, with forms where no carbon is required, or by various office copier machines. Reports may be kept 1, 3, or 5 years or on a perpetual basis with long-term storage on microfilm or in storage boxes located in state archives. In general, retrieval of records beyond 2 years is frequently difficult because of the location of inactive file storage areas.

Current files of reports on public water supplies may be indexed by individual municipal supplies, by county or regional area, or by month. In several states, the individual records for municipal supplies are scattered over the state in the files of branch laboratories assigned the responsibility for examining the municipal water supplies in their geographical area.

PERSONNEL

The size of the laboratory staff required for a given volume of bacteriological examinations may be difficult to predict because of such factors as demands due to other laboratory program needs, availability of laboratory support personnel, and personnel involvement with laboratory administration and clerical duties. An analysis of 1965 to 1970 data on laboratory staffing indicates that 89.0 percent of the central state laboratories had only one to four technicians; 89.3 percent of municipal laboratories employed one to three technicians; and 92.6 percent of private laboratories had only one or two technicians involved in water analysis on a part-time basis. For the number of samples that could be analyzed per technician, data from 10 state laboratories employing the MF procedure was used to estimate an average of 5,400 samples per year. This estimate is about 10 percent higher than the 4,900 samples per year examined, on the average, by technicians using the MPN procedure in 36 other state and branch laboratories. A greater difference in workload would be evident if the numerous related duties (milk and food analyses, record keeping, etc.) common to these state health department laboratories were not involved.

Ideally, the professional staff should include a senior bacteriologist with a major in bacteriology from a recognized college and a MS (or MA) degree or equivalent experience in water bacteriology. As an assistant to the unit chief, the second staff member should have graduated from a recognized college with a major in bacteriology-biology or have equivalent practical experience in water bacteriology. Such employees can carry out or supervise routine test procedures, training activities, consultations on methods and problems, and evaluations of new or routine procedures as needed. Because of the greater number of samples collected during the summer months, qualified temporary help, to work under the direct supervision of the bacteriologists, may be added as required.

Laboratory support personnel, i.e. scientific aids, are also needed to clean glassware and prepare sterile media, sample bottles, and other materials. The specific number of scientific aids required is determined by demands for their services from other laboratory program needs, the volume of disposable plastic items used, the number of water examinations conducted, and the choice and variety of tests performed. Our study of man-power requirements in 18 state laboratories during the period 1965 to 1970 showed that for each staff bacteriologist in the water laboratory, the full-time support of 1.4 scientific aids, assigned to the preparation unit, was needed. In terms of the total number of samples examined each year, these same laboratories required back-up services of one scientific aid for every 6,900 water samples examined per year.

The laboratory staff must also have clerical support to type, file, and distribute copies of reports to the laboratory director, sanitary engineering section, water companies, and private individuals. Laboratory activities also require such additional services as handling telephone messages, preparing correspondence, requisitioning supplies, and compiling semi-annual or annual summaries of laboratory activities. In large laboratory operations, these activities generally require the services of two full-time clerk-typists; in the small municipal laboratory, one clerk-typist should be sufficient.

REFERENCE MATERIAL

A copy of the current edition of *Standard Methods* must be available in the laboratory for immediate use when some aspect of methodology must be reviewed, since this essential reference undergoes substantial revision with each new edition. Some state laboratory systems have prepared excellent methods manuals for distribution to laboratories within their states. These manuals serve as a guide to proper sampling techniques, and provide protocol on sample transit-time restrictions, the use of report forms, laboratory procedures, and data interpretation. This technical information is useful to sanitarians and laboratory personnel in city and county health departments, water works personnel, and institutions involved in the bacteriological examination of water. Concerted effort should be made to periodically up-date these manuals and to circulate them throughout the state to all laboratories. In addition, such references as the EPA manual on microbiological procedures (see reference 36, page 121) on approved protocols and the EPA student training manual (EPA-430/1-74-008, available from NTIS) employed for analysis of municipal effluents should be available and used.

A newsletter, initiated from the office of the state laboratory director on a quarterly basis, can be useful for keeping regional laboratory personnel informed of significant activities related to the mission responsibilities of the laboratory system. The newsletter could also include comments on operating and maintaining laboratory equipment, plus evaluation reports on equipment items for specific laboratory needs that might be purchased in the future.

Reference books that are recommended, but not mandatory, include recent editions of college textbooks on bacteriology, chemistry, statistics, the Merk Index, and commercial application manuals on dehydrated media and testing procedures. Other suggested references, which should be available in the laboratory, include current editions of training manuals acquired through staff participation in state-of-the-art laboratory courses given by the state health department or environmental agency or other specialized courses given by universities. Federal agencies, and commercial interests sponsoring workshops and seminars. Since the science of water bacteriology, chemistry, biology, and sanitary engineering is progressing at a rapid pace, it is essential that professional personnel be given the opportunity to obtain short-term specialized training in new concepts, instrumentation, and methodology.

Several laboratory groups can establish a policy of cooperative sharing of scientific periodicals obtained through personal memberships in various scientific societies. By circulating current journal issues among the staff or by alerting staff members to articles that relate to their specialties, the entire work group can be informed on new research findings. Among the scientific journals that frequently contain articles on water bacteriology are Journal of the Water Pollution Control Federation; Applied Microbiology; Health Laboratory Science; Journal of the American Water Works Association; and Water Research: The International Journal on Water Pollution Research.

LABORATORY FACILITIES

Laboratory space must be adequate to accommodate periods of peak work load. Working space requirements must include sufficient bench-top area for processing samples; storage space for media, glassware, and portable equipment items; floor space for stationary equipment (incubators, water baths, refrigerators, etc.); and an adequate associated area for cleaning glassware and sterilizing materials. The bench-top working area needed for processing samples has been estimated to approximate 4 to 6 linear feet of continuous area per technician. This figure is a practical estimate derived from space requirements observed in various laboratories performing routine analyses. Where more specialized bacteriological examination of water is required, or in laboratories involved in bacteriological research, this space requirement may be inadequate.

The space required for both laboratory work and materials preparation in small water plant laboratories may be consolidated into one room, with the various functions allocated to different sections of the room. In larger water plants, county health department laboratories, and in state and Federal laboratories, the laboratory working area and supporting functions should be in separate rooms but located on the same floor and in proximity to each other. For laboratories engaged in various disciplines—i.e., water, milk, food—work space must be increased proportionally so that water and other samples may be processed as necessary throughout the day without the need to program limited work space and time for one or the other type of sample examination. Where laboratory facilities are limited, the quality of work and the reliability of data may be impaired.

Where possible, media preparation, glassware processing, and sterilization of materials for different laboratory groups in multi-function laboratories should be consolidated. Combining these services results in more economic operation, more efficient use of manpower assigned to these duties, and less duplication of equipment needed for such services (e.g., autoclaves, hot-air sterilizers, automatic glassware washers, automatic pipetting machines, pH meters, balances).

The laboratory should be located in a clean, well-lighted, well-ventilated room (preferably air conditioned) that is reasonably free of dust and draft and not subject to excessive temperature changes. A light intensity of 60 to 100 foot candles is recommended at all working surfaces (1). A bench height of 36 inches provides knee space and convenience for the technician who may choose to stand or sit while performing various tasks. Laboratory benches, 30 inches high should also be provided for use in counting pour plates and MF cultures, in scanning Gram stains, and in recording data on laboratory work sheets. Laboratory table or bench-top working areas should be level to avoid uneven colony distribution over pour plates or over the effective filtration area of MF's. A laboratory sink is essential for the disposal of discarded samples, surplus media, or sample filtrates derived from MF procedures.

Ample cabinets, drawers, and/or shelves should be available in the laboratory for storage and protection of glassware, small laboratory equipment, and other materials, especially when sterilized items are stored for any length of time. The storage area for dehydrated media should not be located near the glassware working area because summer temperatures and humidity may cause deterioration of dehydrated media supplies.

LABORATORY SAFETY

Laboratory safety, which must be an integral and conscious effort in everyday laboratory operations, should provide safeguards to correct facility deficiencies and equipment failures, avoid electric shock, prevent fire, prevent accidental chemical spills, minimize microbiological dangers, and minimize radiation exposures (2-7). Laboratories now are under the Occupational Safety and Health Act or state equivalent safety and health program. Free consultation and advising services from these groups are usually available for safety programs.

Room space must be adequate to avoid storing equipment and supplies along traffic areas that must be accessible to carts, portable equipment, and free movement of technicians. The floors of the laboratory should be clean, dry, and free from projections that might trip personnel or jam cart passage. When floor wax is required, a nonskid wax should be chosen.

Protective maintenance of autoclaves requires periodic inspections by a representative of the manufacturer (see section on Autoclaves in Chapter III). Operating instructions for autoclaves and stills should be posted nearby, particularly if such equipment may be used by inexperienced personnel or on weekends or holidays when those routinely responsible for operation are away.

Electrical service in the laboratory should conform with local, state, or national electrical codes (8). Service feeders must be of adequate size as specified by the applicable electric code and be properly protected from overload by either automatic circuit breakers or fuses. All electrical outlets should be properly grounded using a three-wire ground system. In addition to providing equipment grounds, the three-prong plug orients connections to the electrical wiring so that the hot and neutral side of the equipment circuit always remain at the same potential. In the absence of the three-wire ground system, a separate ground wire, size No. 14 or 16 gauge, must be connected from laboratory equipment to a cold water pipe as a protection from electrical shock. Open wiring should not be used in the laboratory.

All laboratories should have access to both foam and carbon dioxide type fire extinguishers. Foam extinguishers are effective on small fires in ordinary combustible materials and in small quantitites of flammable liquids or grease. Carbon dioxide fire extinguishers must be used where electrical equipment fires occur. These fire extinguishers must be periodically inspected and replaced as necessary. Fire extinguishers should be located either in the laboratory or in a corridor so that a person need not travel more than 50 feet from any point to reach the nearest extinguisher.

Other equipment that should be available in case of fire or chemical accidents includes gas masks, fire blankets, and emergency shower stations. Fire exits from the laboratory must remain clear at all times and not become cluttered with equipment, boxes, or cartons of supplies.

Although the hazards from handling and storing chemicals in the bacteriological laboratory may not be of the magnitude found in the chemistry laboratory, bacteriologists and other laboratory personnel are often unaware of the basic safety rules that must be followed. All chemical containers must be clearly labeled; any materials in unlabeled containers should be carefully discarded. After a reagent has been used, any residual material adhering to the outside of the bottle should be wiped or rinsed off to prevent contact with the hands during future handlings. Flammable solvents should be stored either in an approved solvent storage cabinet or in a well-ventilated area. Avoid storing solvents above eye level in the work area, near open flames, or in refrigerators or cold rooms that also are used to store stock cultures and media. Fumes from leaking containers of organic solvents are often toxic to bacteria. Oxidizing materials such as nitrates and chlorates should be stored in a dry area separate from organic material. When it is necessary to open bottles that may be under pressure (hydrochloric acid or ammonium hydroxide), cover the bottle with a towel to intercept any chemical spray. Bottle carriers should be used when transporting glass bottles containing hazardous chemicals (acids, corrosives, or flammable liquids).

Compressed gas cylinders should be stored and transported with the shipping cap on. Use a wheeled cart to transport large cylinders, and be certain the cylinders are secured at all times. Gas cylinders should be stored and used in an upright position, being fastened securely and well away from any heat source. Before use, double check the identity of the gas cylinder to be certain it is the kind required for the experiment, and always use a reducing valve or preset pressure controller on the cylinder outlet. Do not force connections or use some improvised adaptors.

The microbial agents that might be of potential hazard in the water laboratory are those that could produce disease of varying degrees of severity (as the result of accidental inoculation or injection or other means of cutaneous penetration) but that should be contained by ordinary laboratory techniques (9). Basic dangers associated with microbiological hazards in the laboratory involve (a) hand-mouth contact while handling contaminated laboratory materials and (b) aerosols created by pipetting, centrifuging, or blending samples or cultures and those created by use of inoculating loops (10).

Aerosols can be created by blowing out the last drop from pipettes. Do not mix dilutions by blowing air through a pipet into the culture. When working with grossly polluted water samples, such as sewage or high-density bacterial emulsions, the use of cotton plugs in the mouth end or a rubber bulb attached to the mouth end of the pipet is recommended to prevent the accidental ingestion of sample material. Since untreated waters may contain waterborne pathogens, it is essential that all used pipets be discarded into a jar containing a disinfectant solution for decontamination before these items are returned to personnel responsible for glassware washing. The habit of placing discarded pipets on table tops, laboratory carts, or in sinks without adequate decontamination presents an unnecessary health risk to the laboratory personnel. Quaternary ammonium compounds that include a compatible detergent and solutions of sodium hypochlorites are satisfactory disinfectants for pipet discard jars. The highest concentrations recommended for these commercial products should be used provided this concentration does not cause a loss of markings or fogging of glass pipets. Disinfectant solutions in the discard container should be replaced each morning to ensure maximum disinfection action. Contaminated materials (cultures, samples, used glassware, sereological discards, etc.) must be sterilized by autoclaving before being thrown away or being processed for reuse.

Shattering of culture-containing tubes during centrifugation liberates voluminous quantities of bacterial aerosol in the laboratory (11, 12). Blenders must be leak-proof and tightly covered during operation to prevent creating an aerosol spray that might contaminate technicians stationed some distance away. An investigation of various inoculating loop techniques showed that inserting a hot loop into a flask of broth culture created the greatest hazard in terms of aerosolized bacteria (13). The use of electric heater incineration for sterilizing inoculating loops or needles may be a desirable procedure, but observe caution to avoid a possible electrical shock that could occur if the person holding the loop touches the inside of the heater core while also being grounded (14).

Good personal hygienic practices are important in the control of contact exposures. Frequent disinfection of hands and working surfaces is essential. Smoking, eating, or taking coffee breaks at the work bench should be avoided. Drinking water should be available outside the laboratory, preferably from a foot-operated drinking fountain. The laboratory staff should also be immunized against tetanus and possibly typhoid or other infectious agents that might be under investigation.

Flies and other insect occurrences must be minimized in the laboratory to prevent contamination of sterile equipment, media, samples, and bacterial cultures in addition to the obvious desire to prevent any spread of infectious organisms to the personnel via this vehicle. Control measures must include restriction on food storage in desks and storage cabinets, installation of screens in all windows and outer doors for those laboratories without air-conditioning, and a program of periodic spraying of insecticide along toe-stripping, sink and storage cabinet areas, and utility service channels. Since some laboratories also include a chemistry section that analyzes waters for pesticides, application of insecticides to suppress insect occurrences must be carefully restricted to the immediate areas of the bacteriological laboratory section.

In those laboratories where radioactive chemicals for tracer studies and rapid bacterial detection systems are used, personnel should carry film badges or pocket radiation dosimeters for monitoring individual exposure. Records should be kept of yearly total exposure for each individual staff member. Work areas where radioactive materials are used should be monitored once a week and these readings logged also. Area monitoring should be conducted using a survey instrument (a Geiger-Müller or ionization chamber type) capable of detecting 0.01 milliroentgen per hour, with a maximum of 0.5 milliroentgen per hour at full-scale detection on the lowest setting. Using disposable laboratory items will eliminate many washing problems. Radioactive-contaminated disposable items can be placed in special waste containers, which, when full, can be disposed of by the radiation safety officer. Nondisposable lab-ware contaminated by radioactivity should be held apart from other lab-ware items for suitable cleanup procedures. Where C^{14}-labeled compounds are used, liquid wastes may safely be released into the sanitary sewer provided the quantity released does not exceed an average concentration of 0.02 microcuries per liter. Other radioactive liquid wastes may also be disposed of via the sanitary sewer subject to concentration limits established under Federal regulations (15). Protective plastic or rubber gloves should be worn whenever handling radioactive liquids as a protection for skin cuts or hangnails. When hands are contaminated, they should be thoroughly washed (2 to 3 minutes) in warm water using mild soap or detergent. In no case should abrasive and/or alkaline soap be used. Washing should be repeated several times with the exposed skin area monitored for radioactivity until the hands are decontaminated.

Finally, every laboratory should have a copy of a manual on laboratory safety and a laboratory emergency treatment chart for guidelines in first-aid treatment of accident victims. First aid supplies should be checked frequently to replace out-of-stock items or items that have limited shelf life.

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GUIDELINES ON LABORATORY MANAGEMENT

Laboratory Records

Results assembled and available for inspection .... ________
Data processed rapidly through laboratory and engineering sections ...... ________
Adequate data retention, efficient filing system, and prompt
    channeling of report copies .... ________
Number of tests per year
    MPN Test - Type of sample ________________________________
        Confirmed (+) ____________ (−) ____________ (Total) ____________
        Completed (+) ____________ (−) ____________ (Total) ____________
    MF Test - Type of Sample ________________________________
        Direct Count (+) ____________ (−) ____________ (Total) ____________
        Verified Count (+) ____________ (−) ____________ (Total) ____________

Personnel

Adequately trained or supervised for bacteriological examination of water . ________
Personnel involved:
    Professional staff (total) ________________________________
    Sub-professional support (total) ________________________________
    Clerical assistance (total) ________________________________

Reference Material

Copy of *Standard Methods* (current edition) available in the laboratory ... ________
State or Federal manuals on bacteriological procedures available for staff use ________
State or Federal agency newsletter on laboratory information
    available for staff use .... ________
Scientific journals in water research accessible .... ________

Laboratory Facilities

Laboratory room space and bench-top area adequate for needs
    during peak work periods .... ________
Prep room space adequate and located near laboratory .... ________
Sufficient cabinet space for media, chemicals, glassware,
    and equipment storage .... ________
Facilities clean, with adequate lighting and ventilation, and reasonably
    free from dust and drafts .... ________
Office space and equipment available for processing water examination
    reports and mailing sample bottles .... ________

Laboratory Safety

Personnel and carts permitted mobility without obstructions
    that cause accidents .... ________
Adequately functioning autoclaves and stills, with periodic
    inspection and maintenance .... ________
Electrical service conforms to local, state or National Electrical Codes ... ________
All electrical equipment grounded through three-wire system or
    separate ground to cold water pipe .... ________
Foam-type and carbon dioxide fire extinguishers accessible .... ________
Fire exits from laboratory clear at all times .... ________
Emergency (deluge) shower accessible and functional .... ________
Safety features such as pipet waste jars with disinfectant, centrifuge
    shield, splatter guard, and blender covers employed to avoid
    bacterial aerosols .... ________
Approved practices for handling and disposing of radioactive
    chemicals used in special bacteriological procedures .... ________

First aid supplies available and not out-dated .... ________

Personnel trained to safely handle steam, flames, chemicals, pathogens, etc. ________

Personnel indoctrinated in first aid emergency procedures, fire control, etc. ________

Broken glass, sharp needles, etc., properly handled and disposed of ...... ________

CHAPTER XI
THE NARRATIVE REPORT

PREPARING A NARRATIVE REPORT

Once the on-site evaluation of the laboratory has been completed, including an informal conference summation of the findings, a narrative report must be prepared by the laboratory survey officer to accompany the completed survey form (EPA-103, Bacteriological Survey for Water Laboratories). The primary purpose of this report is to inform Federal and state authorities in the water supply program as to the acceptability of data being developed in the laboratory for use in water quality monitoring. This status report is then further detailed with recommendations directed toward furthering improved data refinement and monitoring effectiveness. Where deviations from *Standard Methods* or the recommended procedures in the EPA Microbiology Methods Manual are observed, the problem should be described with supporting evidence. Recommendations must also include an adequate rationale of the need for change. The technical report must not be used by the laboratory survey officer as a mechanism to express unsupported personal opinions nor should the report be used to promote personal favorite choices of methods, media, instruments, or commercial products without factual data or other evidence to support such claims. The text must be written in clear, concise, precise language. Sheer bulkiness of the report is no criterion of excellence. Finally, the narrative report must be prepared promptly upon laboratory survey officer's return to the duty station while the facts are still readily recalled from notes, survey form, and memory. The Federal Water Supply program recommends the following format:

1. Title

This first section of the report immediately identifies the what, where, when, and by whom for the reader.

Survey Report on the
Bacteriological Examination of Water
at the
(name of laboratory)
(street address)
(City, State, Zip Code)
(date of survey)

by

(name, title, organization,
and address
of reviewing consultant)

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2. Laboratory Certification Status

This section immediately announces the survey officer's decision on the laboratory certification status.
Example:

> The equipment and procedures employed in the bacteriological analysis of water by this laboratory conformed with the provisions of *Standard Methods for the Examination of Water and Wastewater* (current edition) and with the provisions of the Federal *Drinking Water Standards* (see most recent update in the Federal Register), except for the items marked with a cross "X" on the accompanying survey form (EPA-103). Items marked "O" do not apply to the procedures programed in this laboratory. Specific deviations are described, and appropriate remedial action for compliance is given in the following recommendations:

3. Recommendations

List each deviation by item number used on the survey form; describe exact deviation, supplement by tabular data or specific case histories if necessary, and recommend procedural change for compliance with standard procedures.

4. Laboratory Evaluation Program

This section applies only when a Federal or state laboratory program, whose responsibility it is to evaluate other water laboratories within its geographical areas of responsibility, is, itself, being evaluated. All laboratories known to examine water within the geographical area, the nature of their involvement (bacteriological), dates of the most recent laboratory evaluations, and the names of the specific survey officers should be tabulated. Results of a split sampling program for these certified laboratories should also be included in the table whenever this supplemental service is performed. Where the program activity requires the endorsement of new or additional survey officers, a statement of their acceptability should be included in this section. Such endorsements can only be made after the senior evaluation officer in the state or Federal laboratory evaluation service has observed the candidate's technical competence and approach to the assignment during a survey.
Example:

> Ms. O. Serve, Supervising Microbiologist III, is the designated state water laboratory survey officer. During my 2-day conference on laboratory procedures at the Central Laboratory, Ms. Serve demonstrated the qualities of temperament desirable to obtain the cooperation of laboratory personnel in improving their procedures where necessary, without incurring a feeling of resentment. Ms. Serve is familiar with bacteriological indicator concepts, detection methods using multiple tube, membrane filter and pour plate techniques, laboratory ap-

> paratus, media requirements, and analyses of laboratory records. For these reasons, we are pleased to certify Ms. Serve as the State Environmental Protection Agency (or State Health Department) water laboratory survey officer.

5. Remarks

Additional comments on procedures, description of special tests, record systems, equipment, space, and personnel needs may be included under this head. If there are no remarks, delete this section from narrative report.

6. Commendation

If there is administrative protocol or laboratory leadership in the water program deserving special commendation, place such remarks under this head. Such commendation should only be used for outstanding performance and include an adequate description of the impact on the water program. If no commendation is included, delete this section from narrative report.

7. Personnel Certification

Names and titles of personnel together with a general statement of the scope of procedures for which each individual has been approved are listed in this section. Names listed either by rank or in alphabetical order.
Examples:

> Dr. E. Coli, supervising bacteriologist, is approved for the application of multiple tube procedure and membrane filter method for total coliform detection and the standard plate count to the examination of potable water; and the fecal coliform, fecal streptococcus and *Salmonella* techniques to a variety of raw surface and groundwaters used for public water supply intake and treatment.
>
> Ms. C. Water, laboratory technician, is approved for he application of the membrane filter total coliform procedure, and standard plate count examination of potable water.

As an alternative approach, personnel certification may also be given in a blanket approval to the staff, if all are equally knowledgeable and involved in the various water examination procedures.
Example:

> The following laboratory personnel are approved for the application of the membrane filter total coliform procedure (or multiple tube procedure) and the standard plate count to the examination of potable water:
>
> Dr. P. Gram, Supervising Microbiologist
> Mrs. B. Scope, Public Health Bacteriologist
> Mr. M. Filter, Laboratory Technician IV
>
> This staff is also approved for the application of total coliform, fecal coliform, fecal streptococcus, *Pseudomonas aeruginosa*, and *Salmonella* procedures

to a variety of waters including fresh and marine recreational waters, effluents, and stream water quality measurements.

8. Conclusions

Give descriptive conclusions: include recommendations for approval or rejection of the laboratory. Typical conclusions of laboratory quality fall into one of three categories: (a) unqualified acceptance; (b) qualified acceptance; or (c) prohibitive status. Unqualified acceptance is the highest rating given to those laboratories that had no apparent deviations from standard procedures during the period of the on-site survey. Qualified acceptance recognizes some deviations from acceptable procedures—deviations that do not seriously affect the validity of results. The prohibitive rating is given when the laboratory lacks essential equipment, materials, or properly trained personnel, any one or all of which results in major technical deficiencies that grossly affect the validity of laboratory results. Reclassification of a laboratory on prohibitive status will require acquisition of essential laboratory equipment and supplies necessary to perform the bacteriological tests as described in the current editions of *Standard Methods for the Examination of Water and Wastewater* or in the EPA Microbiological Methods Manual, in addition to training the designated laboratory personnel in basic techniques used in water bacteriology. Upon satisfactory completion of these requirements, the laboratory directors should request a resurvey of the laboratory, provided they wish the laboratory data to be used in any official compliance monitoring program.

The specific categories of conclusions can be expressed as:

A. *Unqualified Acceptance*

The procedures and equipment in use at the time of this survey were in compliance with the provisions of *Standard Methods for the Examination of Water and Wastewater* (current edition) and the Federal *Drinking Water Standards* (Federal Register, current revision). Therefore, it is recommended that the results of bacteriological examinations made by this laboratory be accepted as official data defined by the *Safe Drinking Water Act* (Public Law 93-523, Dec. 16, 1974).

B. *Qualified Acceptance*

The procedures and equipment in use at the time of this survey complied in general with the provisions of *Standard Methods for the Examination of Water and Wastewater* (current edition) and the Federal *Drinking Water Standards* (Federal Register, current revision), and with correction of deviations listed, it is recommended that the results of bacteriological examinations made by this laboratory be accepted as official data defined by the *Safe Drinking Water Act* (Public Law 93-523, Dec. 16, 1974).

C. *Prohibitive Status*

The procedures and equipment in use at the time of this survey showed major deviations from the provisions of *Standard Methods for the*

Examination of Water and Wastewater (current edition) and the Federal *Interim Drinking Water Standards* (Federal Register, current revision).

As a result of procedural deficiencies, test sensitivity is below an acceptable level for monitoring potable water quality, established at one coliform organism per ml. The laboratory is, therefore, placed on a prohibitive status for the bacteriological examination of public water supplies as required by the *Safe Drinking Water Act* (Public Law 93-523, Dec. 16, 1974).

Requirements for a reclassification of this laboratory to acceptable compliance will require: (a) acquisition of essential equipment items and supplies; (b) training of designated laboratory personnel in basic techniques used in water bacteriology, followed by; (c) satisfactory compliance in a resurvey of the laboratory to be requested at such time as the laboratory director deems that deficiencies cited in this report have been satisfied.

The narrative report must be signed by the survey officer or consultant who conducted the evaluation and prepared the completed survey form.

PROCESSING THE REPORT

In a cover letter prepared to accompany the report, comments concerning deviations are summarized and the laboratory director is requested to respond promptly, indicating that compliance or corrective actions were taken. Copies of the evaluation report (cover letter, narrative, and survey form) should be forwarded to the appropriate EPA regional office, the state engineering director, and state laboratory director. The original copy should be retained in the office of the laboratory survey officer as part of the file on this program activity.

GUIDELINES ON PREPARING AND PROCESSING A NARRATIVE REPORT

Anatomy of the Technical Report

Report prepared promptly ________
Recommendations include an adequate rationale ________
Devoid of unsupported personal opinion or personal preferences not supported by facts ________
Narrative in clear, concise, precise language ________
Report Format
1. Title (what, where, when, and by whom) ________
2. Lab Certification Status (approved or prohibited) ________
3. Recommendations (deviations described) ________
4. Laboratory evaluation program (program activity described) ________
5. Remarks (suggestions, not deviations, noted) ________
6. Commendation (unusual protocol or leadership noted) ________
7. Personnel Certification (staff capabilities defined) ________
8. Conclusions (data do or do not meet requirements of the *Federal Drinking Water Standards*) ________

Processing the Report

Cover letter sent to laboratory director requesting response ________
Report transmitted included cover letter, narrative, and completed laboratory survey form ________
Copies of the report sent to EPA regional office, state engineering director, and laboratory director ________

GLOSSARY

abatement: The method of reducing the degree or intensity of pollution, also the use of such a method.

acclimation: The physiological and behavioral adjustments of an organism to changes in its immediate environment.

acid: Most commonly refers to a large class of chemicals having a sour taste in water; ability to dissolve certain metals, bases or alkalies to form salts and to turn certain acid-base indicators to their acid form. Characterized by the hydrated H^+ ion.

aeration: The process of adding oxygen to, removing volatile constituents from, or mixing a liquid by intimate contact with air.

aerobe: An organism capable of growing in the presence of oxygen.

aerobic: Description of biological or chemical processes that can occur only in the presence of oxygen.

aerosol: A suspension of liquid or solid particles in the air.

agar: Dried polysaccharide extract of red algae (*Rhodophyceae*) used as a solidifying agent in microbiological media.

algae: Primitive plants, one- or many-celled, usually aquatic and capable of growth on mineral materials via energy from the sun and the green coloring material, chlorophyll.

alkalinity: The sum of the effects opposite in reaction to acids in water. Usually due to carbonates, bicarbonates, and hydroxides; also including borates, silicates and phosphates.

amperometric chlorine residual: A means of determining residual available chlorine with phenyl arsene oxide (PAO) titration using current response as an indicator of equivalence. For wastewater, the PAO preferably is used in excess with iodine backtitration.

anaerobe: An organism capable of growing in the absence of atmospheric oxygen, with essential oxygen being obtained from sulfates, carbonates, or other oxygen-containing compounds.

anaerobic: Life processes or chemical reactions that occur in the absence of oxygen or a condition in which dissolved oxygen is not detectable in the aquatic environment.

anion: A negatively charged ion in water solution. May be a single or a combination of elements, e.g., the Cl^- ion in a water solution of NaCl (common table salt) or $SO_4^=$ ion in a H_2SO_4 (sulfuric acid) solution.

antibiotic: Organic toxins excreted by a microorganism (bacterium or fungus) that inhibits or kills another microorganism.

antibody: A protein molecule formed by the body in response to the presence of an antigen.

antigen: A foreign stimulant (usually a protein) that induces the formation of antibodies in the body.

approved laboratory methods: Approved laboratory methods are those specified in *Standard Methods for the Examination of Water and Wastewater*, prepared and published jointly by the American Public Health Association, the American Water Works Association, and the Water Pollution Control Federation and those specified by the EPA manual *Microbiological Methods for Monitoring the Environment*.

autoclave: An apparatus using steam under pressure for sterilization.

available residual chlorine: Generally refers to that part of the chlorine that will react with ortho tolidine or amperometric tests and exhibits significant germicidal activity.

bacillus: Rod-shaped bacterium; a genus of the family *Bacillaceae*.

bacteria: Primitive organisms having some of the features of plants and animals. Generally included among the fungi. Usually do not contain chlorophyll, hence commonly require preformed organic nutrients among their foods. May exist as single cells, groups, filaments, or colonies.

bactericide: Any component that will kill or destroy bacteria.

bacteriophage: A virus that infects bacteria and effects lysis of bacterial cells.

bacteriostatic: A condition during which the normal metabolic functions of bacteria are arrested until favorable conditions are restored.

biological oxidation: The process by which bacterial and other microorganisms feed on complex organic materials and decompose them. Self-purification of waterways and activated sludge and trickling filter waste water treatment processes depend on this principle. The process is also called biochemical oxidation.

BOD$_5$: The amount of dissolved oxygen consumed in 5 days by biological processes breaking down organic matter in an effluent.

buffer action: An action exhibited by certain chemicals that limits the change in pH upon addition of acid or alkaline materials to a medium or other fluid. In surface water, the primary buffer action is related to carbon dioxide, bicarbonate, and carbonate equilibria.

capsule: A gelatinous envelope or slime layer surrounding the cell wall of certain microorganisms.

carrier: A person in apparently good health who harbors a pathogenic microorganism.

catalyst: A substance that influences the rate of chemical change but either remains unchanged during the reaction or is regenerated thereafter.

centigrade: (Celsius) A temperature measurement in which the freezing point of pure water at sea level is designated as 0°C and the boiling point designated as 100°C.

cfs: Cubic feet per second, a measure of the amount of water passing a given point.

chloramines: Products of the combination of chlorine and ammonia. Commonly classified as combined available chlorine.

chlorination: The application of chlorine to water or wastewater for the purposes of disinfection, oxidation, odor control, or other effects. Pre-chlorination — before treatment; post-chlorination — after treatment; in-process chlorination — during treatment.

chlorine demand: The difference between applied chlorine and residual available chlorine in aqueous media under specified conditions and contact time. Chlorine demand varies with dosage, time, temperature, nature, and amount of the water impurities.

coagulant: A chemical, or chemicals, which when added to water suspensions will cause finely dispersed materials to gather into larger masses of improved filterability, settleability, or drainability.

coagulation: The clumping of particles to settle out impurities; often induced by chemicals such as lime or alum.

coccus: A spherical bacterium.

coliform group: A group of bacteria that inhabits the intestinal tract of man, warm-blooded animals; may be found in plants, soil, air, and the aquatic environment. Includes aerobic and facultative gram negative nonspore forming bacilli that ferment lactose with gas formation.

colloid, colloidal state: A state of suspension in which the particulate or insoluble material is in a finely divided form that remains dispersed in the liquid for extended time periods. Usually cloudy or turbid suspensions requiring flocculation before clarification.

colony: A macroscopic mass of microorganisms growing together, the cells of which have a common origin; often used in a limited sense to refer to bacterial masses growing on a solid medium.

combined available chlorine: Generally refers to chlorine-ammonia compounds exhibiting a slower reaction with ortho tolidine, determinable with phenyl arsene oxide after addition of potassium iodide under acid conditions; usually requires higher concentration and longer time to kill microorganisms in comparison with free available chlorine.

communicable: Pertaining to a disease whose causative agent is readily transferred from one person to another.

contamination: A general term referring to the introduction of materials into water that make the water less desirable for its intended use. Also introduction of undesired substances into air, solutions, or other defined media (chemical or biological).

counterstain: A background stain applied to stained material to increase contrast.

criterion (pl. criteria): Some physical, chemical, or biological characteristic that can be measured. Commonly used as a basis for standards.

cross connection: In plumbing, a physical connection between two different water systems, such as between potable and polluted water lines.

deionized water: Water that has been treated by ion exchange resins or compounds to remove cations and anions present in the form of dissolved salts.

desalinization: Salt removal from sea or brackish water.

detritus: The heavier material moved by natural flow, usually along the channel bed. Sand, grit, or other coarse material.

differential medium: Medium developed to elicit a specific characteristic of an organism or group of organisms.

digestion: The biochemical decomposition of organic matter. Digestion of sewage sludge takes place in tanks where the sludge decomposes and results in partial gasification, liquefaction, and mineralization of pollutants.

disinfection: Effective killing by chemical, radiation, or physical processes of all organisms capable of causing infectious disease. Chlorination is the disinfection method commonly employed in water and sewage treatment processes.

dissolved oxygen (DO): The oxygen dissolved in water or sewage. Adequately dissolved oxygen is necessary for the life of fish and other aquatic organisms and for the prevention of offensive odors. Low dissolved oxygen concentrations generally are due to discharge of excessive organic solids having high BOD and are the result of inadequate waste treatment.

distilled water: A purified water resulting from heat vaporization followed later by vapor condensation to separate the water from nonvolatile impurities.

drinking water standards: A list of standards prescribed for potable water acceptable for public consumption. The standards concern sources, protection, and bacteriological, biological, chemical, and physical criteria—some mandatory, some desired.

ecology: The interrelationships of living things to one another and to their environment or the study of such interrelationships.

effluent: Sewage, water, or other liquid, partially or completely treated or in its natural state, flowing from a reservoir, basin, or treatment plant into receiving streams or marine coastal waters.

endemic: Peculiar to or occurring constantly in a community.

endogenous metabolism: A diminished level of metabolism in which various materials previously stored by the cells are oxidized.

endotoxin: A toxin produced in an organism and liberated only when the organism disintegrates.

enteric organisms: Those organisms commonly associated with the intestinal tract of warm-blooded animals.

epidemiology: The study of diseases as they affect populations.

equivalent terms:

Exponential Value	American System	Symbol	British System	Symbol
1×10^{-6}	parts per million	ppm	parts per million	ppm
1×10^{-9}	parts per billion	ppb	parts per milliard	ppm
1×10^{-12}	parts per trillion	ppt	parts per billion	ppb

estuaries: Areas where the fresh water meets salt water. For example, at bays, mouths of rivers, salt marshes, and lagoons.

eutrophic lakes: Shallow lakes, weed-choked at the edges and very rich in nutrients. The water is characterized by large amounts of algae, low water transparency, low dissolved oxygen and high BOD.

eutrophication: An action involving the aging of lakes; characterized by nutrient enrichment and increasing growth of plant and animal organisms. The net effect is to decrease depth until the lake becomes a bog and eventually dry land. Man-made pollution tends to hasten the process.

facultative bacteria: Bacteria that can adapt themselves to growth and metabolism under aerobic or anaerobic conditions. Many organisms of interest in wastewater stabilization are among this group.

fahrenheit: A temperature scale in which pure water at sea level freezes at 32°F and boils at 212°F.

fastidious organism: An organism that is difficult to isolate or cultivate on ordinary culture.

fecal coliforms: A subgroup of coliform bacteria that has a high positive correlation with fecal contamination associated with all warm-blooded animals. These organisms can ferment lactose at 44.5°C and produce gas in a multiple tube procedure (EC confirmation) or acidity in the membrane filter procedure (M-FC medium).

fecal streptococci: Bacterial indicators of fecal pollution whose normal habitat is the intestinal tract of man and other warm-blooded animals. Species and their varieties of particular interest include: *S. faecalis*, *S. faecalis* var. *liquefaciens*, *S. faecalis* var. *zymogenes*, *S. durans*, *S. faecium*, *S. bovis*, and *S. equinus*.

fermentation: A form of respiration by organisms that requires little or no free oxygen, yields alcohol and carbon dioxide as end products, and releases only part of the food energy available; e.g., the conversion of sugars into alcohol by enzymes from yeasts.

filamentous: Characterized by threadlike structures.

filter: A porous media through which a liquid may be passed to effect removal of suspended materials. Filter media may include paper, cloth, sand, prepared membranes, gravel, asbestos fiber, or other granular or fibrous material.

filtrate: Liquid that has passed through a filter.

filtration rate: A rate of application of water or wastewater to a filter. Commonly expressed in million gallons per acre per day or gallons per square foot per minute.

flagellum: A flexible, whiplike appendage on some bacterial cells; used as an organ of locomotion.

floc: Gelatinous or amorphous solids formed by chemical, biological, or physical agglomeration of fine materials into larger masses that are more readily separated from the liquid.

free available chlorine: Generally refers to that chlorine existing in water as the hypochlorous acid. Characterized by rapid color formation with ortho tolidine. Can be titrated in neutral solution with phenyl arsene.

fungi: Simple or complex organisms without chlorophyll. The simpler forms are one-celled; higher forms have branched filaments and complicated life cycles. Examples are molds, yeasts, and mushrooms.

germicide: A chemical agent that kills microorganisms.

Gram stain: A differential stain by which bacteria are classed as Gram-positive or Gram-negative depending upon whether they retain or lose the primary stain (crystal violet) when subjected to treatment with a decolorizing agent.

groundwater: The supply of freshwater under the earth's surface in an aquifer or soil that forms a natural water resource.

growth curve: Graphic representation of the growth (population changes) of bacteria in a culture medium.

habitat: The natural environment of an organism.

hardness: Commonly refers to chemicals interfering with soap action or producing scale in boilers or heating units. Specifically refers to calcium and magnesium salts such as bicarbonate, carbonates, chlorides, and nitrates, sometimes includes iron, aluminum and silica.

humus: A brown or black complex and variable material resulting from decomposition of plant or animal matter.

hydrostatic head: The pressure exerted by a given height of liquid above a given datum point. May be listed in feet of head, pounds per square inch, or other criteria.

IMViC test: A collection of tests used to differentiate *Escherichia* from *Aerobacter*. IMViC stands for *I*ndole, *M*ethyl Red, *V*oges-Proskauer, and *C*itrate. The "i" is for pronunciation convenience only.

indicator: A substance that changes color as conditions change; e.g., pH indicators reflect changes in acidity or alkalinity. Redox indicators respond to changes in reduction-oxidation potential.

infection: Introduction of a foreign organism that can multiply and produce a resulting change from normal.

influent: Material entering a process unit or operation.

inhibition: Prevention of growth or multiplication of microorganisms.

inoculum: A concentration of microorganisms added to a medium to initiate a growth response.

inorganic: Being composed of material other than plant or animal materials. Forming or belonging to the inanimate world.

interstate carrier water supply: A water supply whose water may be used for drinking or cooking purposes aboard common carriers (planes, trains, buses, and ships) operating interstate. Interstate carrier water supplies are regulated by the Federal government.

interstate waters: According to law, waters defined as: (1) rivers, lakes, and other waters that flow across or form a part of State or international boundaries; (2) waters of the Great Lakes; (3) coastal waters, the scope of which has been defined to include ocean waters seaward to the territorial limits and waters along the coastline (including inland streams) influenced by the tide.

leaching: The process by which soluble materials in the soil, such as nutrients, pesticide chemicals, or contaminants, are washed into a lower layer of soil or are dissolved and carried away by water.

medium (pl. media): Any substance that supports the growth and multiplication of microorganisms.

membrane filter (MF): A flat, highly porous, flexible plastic disc, commonly about 0.15 mm in thickness and 47-50 mm in diameter. Membrane filters with a pore size of 0.45μ are used in water microbiology to entrap organisms from a sample. With the use of selected media, incubation time, and choice of temperature, they permit direct enumeration by colony count of selected organisms.

meniscus: The curved upper surface of a liquid in a tube that is concave upward when the containing walls are wetted by the confined liquid and convex upward when they are not.

mesophillic: Organisms capable of optimum metabolic activities at temperatures from about 80° to 110°F (26° to 42°C).

metabolite, essential: A substance whose presence in very low concentration (micrograms per milliliter or below) must be supplied from an external source so that the organism may carry out its normal functions or so that a specific biochemical reaction may be allowed to proceed.

meter: The length of a reference platinum bar used as a standard unit of measurement of length in the metric system; 1 meter = 39.37 inches.

mg/l: Milligrams per liter; a unit of concentration on a weight/volume basis. Equivalent to ppm when the specific gravity of the liquid is 1.0.

micro: 1/1,000,000 of a unit of measurement, such as microgram, microliter.

milli: An expression used to indicate 1/1000 of a standard unit of weight, length or capacity (metric system):

Milliliter	(ml)	1/1000 liter	(l)
Milligram	(mg)	1/1000 gram	(g)
Millimeter	(mm)	1/1000 meter	(m)

mixing zone: An area where two or more substances of different characteristics blend to form a uniform mixture; i.e., chlorine application, heated water, or other discharged materials entering a water mass will show significant differences of chlorine residual, temperature, or other criteria. These differences depend on the sampling location throughout the mixing zone and approach uniform results with respect to lateral, longitudinal, and vertical sampling positions when mixing has been completed.

moisture content: The content of water in some material. Commonly expressed in percentage of moisture in soil, sludge, or feces.

most probable number (MPN): A statistical method of determining microbial populations. A multiple dilution tube technique is utilized with a standard medium and observations are made for specific individual tube effects. Resultant coding is translated by mathematical probability tables into population numbers.

nitrification: The biological oxidation of ammonia to nitrate.

normality: (a) A means of expressing the concentration of a standard solution in terms of the gram equivalents of reacting substances per liter. (b) Generally expressed as a decimal fraction, such as 0.1 or 0.02 N.

nutrients: (a) Anything essential to support life. (b) Include many common elements and combinations of them. The major nutrients include carbon, hydrogen, oxygen, nitrogen, sulfur, and phosphorus. (c) Nitrogen and phosphorus are of major concern because they tend to recycle and are difficult to control.

organic: Substances formed as a result of living plant or animal organisms. Generally contain carbon as a major constituent.

organic chloride: Compounds containing chlorine in combination with carbon, hydrogen, and certain other elements.

ortho tolidine chlorine test: The dye ortho tolidine, under highly acid conditions, produces a yellow color proportional in intensity to the concentration of available residual chlorine and certain other oxidants or interfering materials.

outfall: The mouth of a sewer, drain, or conduit where an effluent is discharged into the receiving waters.

oxidation: Chemically, the addition of oxygen, removal of hydrogen, or the removal of electrons from an element or compound.

parasite: An organism that lives in or on another organism and results in varying degrees of harm or damage.

particulates: Detectable solid material dispersed in a gas or liquid. Small-sized particulates may produce a smoky or hazy appearance in a gas and a milky or turbid appearance in a liquid. Larger particulates are more readily detected and separated by sedimentation or filtration.

pasteurization: Use of heat for a prescribed period of time to reduce the total number of microorganisms, especially pathogenic or otherwise undesirable forms.

pathogen: An organism capable of eliciting disease symptoms in another organism.

Petri dish: Double glass or plastic dish used to cultivate microorganisms.

pH: An index of hydrogen ion activity. Defined as the negative logarithm (base 10) of H^+ ion concentration at a given instant. On a scale of 0 to 14, pH 7.0 is neutral; pH less than 7.0 indicates a predominance of H^+ or acid ions; pH greater than 7.0 indicates a predominance of OH^- or alkaline ions.

pollutant: Dredged spoil, solid waste, incinerator residue, sewage, garbage, sewage sludge, munitions, chemical waste, biological materials, radioactive materials, heat, wrecked or discarded equipment, rock, sand, and industrial, municipal, and agricultural waste discharged into water.

pond: A basin or catchment for retaining water used for equalization, stabilization, or other purposes. Commonly less than 5 feet deep.

potable water: Water suitable (from both health and aesthetic considerations) for drinking or cooking purposes.

ppm (parts per million): A unit of concentration signifying parts of some substance per million parts of dispersing medium. Equivalent numerically to mg/l when the specific gravity of the solution is 1.0.

precipitate: The formation of solid particles in a solution, or the solids that settle as a result of chemical or physical action that caused the solids to suspend from solution

pressure: The total load or force acting upon a surface. In hydraulics, the term commonly means pounds per square inch of surface, or kilograms per square cm, above atmospheric pressure on site. (Atmospheric pressure at sea level is about 14.7 pounds per square inch.)

primary effluent: Effluent from a sewage treatment process that provides partial removal of sewage solids by physical methods so that 1 liter of the effluent does not contain more than 1 ml of settleable solids as determined by an approved laboratory method.

proteins: Naturally occurring compounds containing carbon, hydrogen, nitrogen, and oxygen, with smaller amounts of sulfur and phosphorus and trace components essential to living cells.

protozoa: Single-cell or multiple-cell organisms, such as amoeba, celiates, and flagellates. Commonly aquatic and generally deriving most of their nutrition from preformed organic food.

psychrophilic organisms: Low-temperature-loving organisms, or organisms having a competitive advantage over other organisms at lower temperatures, i.e., from about 10°C downward to the freezing point.

public water supply: A water supply with at least 15-service connections on the distribution network or a supply regularly serving at least 25 individuals. This system includes the water works and auxiliaries for collection, treatment, storage, and distribution of the water from the sources of supply to the free-flowing outlet of the ultimate consumer.

pure culture: A culture containing only one species of organism.

putrefaction: Biological decomposition of organic matter with the formation of ill-smelling products, such as hydrogen sulfide amines, mercaptans; associated with anaerobic conditions.

qualitative: Defines a procedure for detecting the occurrence of organisms or chemical entities in water; applied to *nonmeasurable* aspects.

quantitative: Defines a procedure or object in terms of its *measureable* aspects or characteristics; implies the use of mathematics, especially statistics.

receiving waters: Rivers, lakes, oceans, or other bodies that receive treated or untreated waste waters.

reclaimed waste water: Waters originating from sewage or other waste that have been treated or otherwise purified to permit direct beneficial reuse or to allow reuse that would not otherwise occur.

reservoir: A pond, lake, tank, or basin, natural or man-made, used for the storage, regulation, and control of water.

river basin: The total area drained by a river and its tributaries.

salt: A chemical compound formed as a result of the interaction of an acid and an alkali (base). The most common salt is sodium chloride formed from hydrochloric acid and sodium hydroxide. This ionizes in water solution to form sodium and chloride ions.

saprophytic: Organisms feeding or growing on dead or decaying organic matter. Organisms that utilize nonliving organic matter as a food.

saturation: Commonly refers to the maximum amount of any material that can be dissolved in water or other liquid at a given temperature and pressure. For oxygen, this commonly refers to a percentage saturation in terms of the saturation value, such as about 9 mg 0_2 per liter at 20°C.

screen: A device with openings, generally having a relatively uniform size, that permits liquid to pass but retain larger particles. The screen may consist of bars, coarse to fine wire, rods, gratings, paper, membranes, etc., depending upon particle size to be retained.

sedimentation: The process of subsidence and deposition of suspended matter from wastewater by gravity. Also called clarification, settling.

sewage: Liquid or solid refuse (domestic and industrial wastes) carried off in sewers.

sewage slimes: Consisting of organisms growing on wastewater nutrients and forming mucilaginous films, streamers, or clumps. May consist of bacteria, molds, protozoa, or algae.

sewer: A pipe or conduit, generally covered, for the purposes of conveying wastewaters from the point of origin to a point of treatment or discharge.

sludge: Accumulated or concentrated solids from sedimentation or clarification of wastewater. Contains varying proportions of solids in wastewater depending upon source, process, and nature.

sludge banks: An accumulation of solids, including silt, mineral, organic, and cell mass particulate material, that is produced in an aquatic system characterized by low current velocity. Generally refers to gross deposits of appreciable depth.

sludge cake: The solids remaining after dewatering sludge by vacuum, filtration, or sludge drying beds. Usually forkable or spadable, with a water content of 30 to 80%. Also may occur on the boundaries of surface water.

smear: A thin layer of material, e.g., bacterial culture, spread on a glass slide for microscopic examination. Also referred to as a *film*.

solution: a) A homogenous mixture of gas, liquid, or solid in a liquid that remains clear indefinitely.

b) Generally an atomic, ionic, or molecular dispersion in a liquid (may be colored).

c) A water solution of dissolved material.

specific gravity (Sp. Gr.): a) The weight of a material per unit volume in reference to the weight of water at maximum density.

b) Water at 4°C has a weight of 1 gram per ml. The weight ratio of any substance divided by the weight of water is the specific gravity.

spore: A reproductive unit, lacking a preformed embryo, that is capable of germinating directly to form a new individual. A resistant body formed by certain microorganisms; a resistant resting cell; a primitive unicellular reproductive body.

stabilization: (a) The activity proceeding along the pathway to stability. (b) In organic wastes, generally refers to oxidation via biochemical pathways and conversion to gaseous or insoluble materials that are relatively inert to further change.

stain: A dye used to color microorganisms; used an an aid to visual inspection.

standard: A measurement limit set by authority. Having qualities or attributes required by law and defined by minimum or maximum limits of acceptability in terms of established criteria or measurable indices.

standard methods: Methods of analysis prescribed by joint action of American Public Health Association, American Water Works Association, Water Pollution Control Federation, or U.S. Environmental Protection Agency. Methods accepted by authority.

standard plate count: A measure of the general bacterial population in potable water and swimming pool water using standard plate count agar, 48-hour incubation, and 35°C incubation temperature. The incubation time of standard plate counts of bottled water, done as for potable water supplies, is extended to 72 hours because of the slow generation times for organisms in this water environment.

sterilization: The process of making a medium free of living organisms such as by killing them, filtering them through a porous medium fine enough to be a barrier to the passage of organisms, etc.

stock cultures: Known species of microorganisms maintained in the laboratory for various tests and studies.

stormwater: The runoff of rain and melted snow into the natural drainage pattern.

strain: A pure culture of microorganisms composed of the descendants of a single isolate.

substrate: (a) Any substance used as nutrient by a microorganism. (b) The liquid in an activated sludge aeration tank.

supernate: The liquid over a precipitate or sediment; the fluid remaining after removal of suspended matter.

suspended solids: The concentration of insoluble materials suspended or dispersed in waste or used water. Generally expressed in mg per liter on a dry weight basis. Usually determined by filtration methods.

symbiosis: The living together of two or more organisms in a mutually beneficial state.

synergism: The ability of two or more organisms to bring about changes (usually chemical) that neither can accomplish alone.

thermal pollution: Degradation of water quality by the introduction of a heated effluent. Primarily a result of the discharge of cooling waters from industrial processes, particularly from electrical power generation. Even small deviations from normal water temperatures can affect aquatic life. Thermal pollution usually can be controlled by cooling towers.

thermophilic: High-temperature-loving organisms. Generally considered to include organisms having a favorable competitive advantage at temperatures above 110°F or 42°C.

titration: The careful addition of a standard solution of known concentration of reacting substance to an equivalence point to estimate the concentration of a desired material in a sample.

TOC: Total organic carbon. A test expressing wastewater contaminant concentration in terms of the carbon content.

total solids: Refers to the solids contained in dissolved and suspended form in water. Commonly determined on a weight basis by evaporation to dryness.

ultraviolet rays: Radiations in the part of the spectrum having wavelengths from about 3,900 Angstrom to about 200 Angstrom.

velocity (flow): A rate term expressed in terms of linear movement per unit of time. Commonly expressed in ft per sec (English) or cm per sec (metric).

virulence: The capacity of a microorganism to produce disease.

virus: An obligate intracellular parasitic microorganism smaller than bacteria. A term generally used to designate organisms that pass filtration media capable of removing bacteria. Technically described as a collective term covering disease stimuli held by some to be living organisms and by others to be nucleic acids capable of reproduction and growth.

Voges-Proskauer reaction: A test (VP test) for the presence of acetylmethylcarbinol to assist in distinguishing between species of the coliform group.

volatile acids: A group of low-molecular-weight acids, such as acetic and propionic, that are distillable from acidified solution.

volatile material: a) Descriptive of chemicals having a vapor pressure low enough to evaporate from water readily at normal temperatures. b) With reference to dry solids, the term includes loss in weight upon ignition at 600°C.

wastewater: Refers to the used water of a community. Generally contaminated by the waste products from household, commercial, or industrial activities. Often contains surface wash, storm water, and infiltrations water.

water pollution: The addition of sewage, industrial wastes, or other harmful or objectionable material to water in concentrations or in sufficient quantities to result in measurable degradation of water quality.

water quality criteria: The levels of pollutants that affect the suitability of water for a given use. Generally, water use classification includes: public water supply, recreation, propagation of fish and other aquatic life, agricultural use, and industrial use.

water quality standard: A plan for water quality management containing four major elements: the use (recreation, drinking water, fish and wildlife propagation, industrial, or agricultural) to be made of the water; criteria to protect those uses; implementation plans (for needed industrial-municipal waste treatment improvements) and enforcement plans; and an anti-degradation statement to protect existing high quality waters.

watershed: The area drained by an entire river system, including tributary streams and intermittent creeks.

water supply system: The system for the collection, treatment, storage, and distribution of potable water from the sources of supply to the consumer.

water table: The upper level of groundwater.

zoogloea: A jelly-like matrix developed by certain microorganisms at some stage in their life cycle. Commonly associated with sludge flocculation in biochemical treatment operations.

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16. ABSTRACT

The material included in this Handbook is designed and intended to provide a comprehensive source of information and reference for the evaluation of laboratories involved in bacteriological testing of potable water supplies and their sources. All aspects of the laboratory operation are considered--material and media preparation, equipment needs and specifications, sample collection and handling, bacteriological methodology, quality control considerations, laboratory management, and the qualifications and responsibilities of the survey officer. The purpose of the Handbook is to assist the laboratory survey officer, laboratory director, and senior bacteriologist in charge of the water program to evaluate the many aspects of the laboratory that are involved in attaining reliable data.

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